(54) Title: PROTEGRINS

(57) Abstract

Cationic antimicrobial and virus-neutralizing peptides having 16 to 18 amino acids and comprising 0-4 cysteines are provided as five native protegrins isolated from porcine leukocyte granules having two cystine bridges or as various protegrin analogs having no, or a single, cystine bridge. Native protegrins have, and analogs may have, carboxyl-terminal amidation and analogs may optionally be prepared in amino-terminal acylated and/or cysteine-stabilized and/or carboxyl-terminal esterified forms. Any of the 1-4 native cysteines may be replaced with a hydrophobic or a small amino acid and various substituents are disclosed f r the remaining 12-16 positions. Recombinant host cells and methods for production are disclosed, as well as pharmaceutical compositions, compositions for agricultural application, and methods for bacteriostatic, virus-neutralizing, and endotoxin-inactivating use of native protegrins and their analogs.

PROTEGRINS

Technical Field

The invention relates to the field of antibiotic
peptides. In particular, the invention concerns short
10 peptides, some of which are isolated from porcine
leukocytes, that have a wide range of antimicrobial
activities.

Background Art

15   One of the defense mechanisms against infection by both
animals and plants is the production of peptides that have
antimicrobial and antiviral activity. Various classes of
these peptides have been isolated from tissues both of
plants and animals. One well known class of such peptides
20 is the tachyplesins which were first isolated from the
hemocytes of the horseshoe crab as described by Nakamura, T.
et al. J Biol Chem (1988) 263:16709-16713. This article
described the initial tachyplesin isolated, Tachyplesin I,
from the Japanese species. Tachyplesin I is a 17-amino acid
25 amidated peptide containing four cysteine residues providing
two intramolecular cystine bonds. A later article by this
group, Miyata, T. et al. J Biochem (1989) 106:663-668,
reports the isolation of a second tachyplesin, Tachyplesin
II, consisting of 17 residues amidated at the C-terminus,
30 also containing four cysteine residues and two
intramolecular disulfide bonds. Two additional 18-mers,
called polyphemusins, highly homologous to Tachyplesin II
and containing the same positions for the four cysteine
residues, were also isolated from the American horseshoe
35 crab. Polyphemusin I and Polyphemusin II differ from each
other only in the r placement of one arginine residue by a
lysine. All of the peptides were described as having
antifungal and antibacterial activity. A later article by

Murakami, T. *et al.* <u>Chemotherapy</u> (1991) <u>37</u>:327-334,
describes the antiviral activity of the tachyplesins with
respect to vesicular stomatitis virus; Herpes Simplex Virus
I & II, Adenovirus I, Reovirus II and Poliovirus I were
5   resistant to inactivation by Tachyplesin I. Morimoto, M. *et*
al. <u>Chemotherapy</u> (1991) <u>37</u>:206-211, found that Tachyplesin I
was inhibitory to Human Immunodeficiency Virus. This anti-
HIV activity was found also to be possessed by a synthetic
analog of Polyphemusin II as described by Nakashima, H. *et*
10 *al.* <u>Antimicrobial Agents and Chemotherapy</u> (1992) 1249-1255.
Antiviral peptides have also been found in rabbit leukocytes
as reported by Lehrer, R.I. *et al.* <u>J Virol</u> (1985) <u>54</u>:467-
472.

        Other important classes of cysteine-containing
15 antimicrobial peptides include the defensins, β-defensins
and insect defensins. The defensins are somewhat longer
peptides characterized by six invariant cysteines and three
intramolecular cystine disulfide bonds. Defensins were
described by Lehrer, R.I. *et al.* <u>Cell</u> (1991) <u>64</u>:229-230;
20 Lehrer, R.I. *et al.* <u>Ann Rev Immunol</u> (1993) <u>11</u>:105-128. A
review of mammalian-derived defensins by Lehrer, R.I. *et al.*
is found in <u>Annual Review Immunol</u> (1993) <u>11</u>:105-128; three
patents have issued on the defensins: U.S. 4,705,777; U.S.
4,659,692; and U.S. 4,543,252. Defensins have been found in
25 the polymorphonucleated neutrophils (PMN) of humans and of
several other animals, as well as in rabbit pulmonary
alveolar macrophages, and in murine small intestinal
epithelial (Paneth) cells and in corresponding cells in
humans.

30   β-Defensins are found in bovine respiratory epithelial
cells, bovine granulocytes and avian leukocytes. See
Selsted, M.E. *et al.* <u>J Biol Chem</u> (1993) <u>288</u>:6641-6648 and
Diamond, G. *et al.* <u>Proc Natl Acad Sci</u> (USA) (1991) <u>88</u>:3952-
3958. Insect defensins have been reported by Lambert, J. *et*
35 *al.* <u>Proc Natl Acad Sci</u> (USA) (1989) <u>88</u>:262-265.

        Antifungal and antibacterial peptides and proteins have
also been found in plants (Broekaert, W.F. *et al.*

<u>Biochemistry</u> (1992) <u>31</u>:4308-4314) as reviewed by
Cornelissen, B.J.C. *et al.* <u>Plant Physiol</u> (1993) <u>101</u>:709-712.
Expression systems for the production of such peptides have
been used to transform plants to protect the plants against
5   such infection as described, for example, by Haln, R. *et al.*
<u>Nature</u> (1993) <u>361</u>:153-156.

The present invention provides a new class of
antimicrobial and antiviral peptides, designated
"protegrins" herein, representative members of which have
10   been isolated from porcine leukocytes. These peptides are
useful as antibacterial antiviral and antifungal agents in
both plants and animals.

The isolation of the protegrin peptides of the
invention was reported by the present applicants in a paper
15   by Kokryakov, V.N. *et al.* <u>FEBS</u> (1993) <u>337</u>:231-236 (July
issue). A later publication of this group described the
presence of a new protegrin, whose sequence, and that of its
precursor, was deduced from its isolated cDNA clone. Zhao,
C et al, <u>FEBS Letters</u> (1994) <u>346</u>:285-288. An additional
20   paper disclosing cationic peptides from porcine neutrophils
was published by Mirgorodskaya, O.A. *et al.* <u>FEBS</u> (1993)
<u>330</u>:339-342 (September issue). Storici, P. *et al.* <u>Biochem
Biophys Res Comm</u> (1993) <u>196</u>:1363-1367, report the recovery
of a DNA sequence which encodes a pig leukocyte
25   antimicrobial peptide with a cathelin-like prosequence. The
peptide is reported to be one of the protegrins disclosed
hereinbelow. Additional publications related to protegrins
are Harwig, S.S.L., et al. <u>J. Peptide Sci.</u> (1995) in press;
and Zhao, C., et al. FEBS-MS MB-283 (1995) in press.

30   The protegrins of the invention have also been found to
bind to endotoxins -- i.e., the lipopolysaccharide (LPS)
compositions derived from gram-negative bacteria which are
believed responsible for gram-negative sepsis. This type of
sepsis is an extremely common condition and is often fatal.
35   Others have attempted to design and study proteins which
bind LPS/ ndotoxin, and illustrative reports of these
attempts appear in Rustici, A. *et al.* <u>Science</u> (1993)
<u>259</u>:361-364; Matsuzaki, K. *et al.* <u>Biochemistry</u> (1993)

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32:11704-11710; Hoess, A. *et al.* EMBO J (1993) 12:3351-3356;
and Elsbach, P. *et al.* Current Opinion in Immunology (1993)
5:103-107. The protegrins of the present invention provide
additional compounds which are capable of inactivating of
5   LPS and ameliorating its effects.

        In addition to the foregoing, the protegrins of the
invention are effective in inhibiting the growth of
organisms that are associated with sexually transmitted
diseases. It is estimated that 14 million people world-wide
10   are infected with HIV and that millions of women sustain
pelvic inflammatory disease each year. *Chlamydia
trachomatis* and *Neisseria gonorrhoeae* cause over half of
this inflammatory disease although *E. coli, Mycoplasma
hominis* and other infectious microorganisms can also be
15   responsible. Pathogens include viral, bacterial, fungal and
protozoan pathogens. It is especially important that the
antibiotics used to combat these infections be effective
under physiological conditions. The protegrins of the
present invention offer these properties.
20

Disclosure of the Invention

        In one embodiment, the invention is directed to
peptides of 16-18 amino acid residues characterized by four
invariant cysteines and either by a characteristic pattern
25   of basic and hydrophobic amino acids and/or being isolatable
from animal leukocytes using the method of the invention.
In a second embodiment, the invention is directed to the
above peptides wherein 1-4 of these cysteines is replaced by
a hydrophobic or small amino acid. All of these peptides
30   can be produced synthetically and some can be produced
recombinantly or can be isolated from their native sources
and purified for use as preservatives or in pharmaceutical
compositions in treating or preventing infection in animals.
Alternatively, the peptides can be formulated into
35   compositions which can be applied to plants to protect them
against viral or microbial infection. In still another
approach, the DNA encoding the peptides can be expressed *in
situ*, in animals or preferably in plants, to combat

infections. The peptides are also useful as standards in antimicrobial assays and in binding endotoxins.

Accordingly, in one aspect, the invention is directed to a purified and isolated or recombinantly produced
5   compound of the formula

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_7-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \quad (1)$$

and the N-terminal acylated and/or C-terminal amidated
10 or esterified forms thereof, which is either in the optionally -SH stabilized linear or in a cystine-bridged form

wherein A_1 is a basic amino acid;

each of A_2 and A_3 is independently a small amino acid;
15   each of A_5, A_7, A_{14} is independently a hydrophobic amino acid;

A_4 is a basic or a small amino acid;

each of A_9, A_{12} and A_{16} is independently a basic, a hydrophobic, a neutral/polar or a small amino acid;
20   each of A_{10} and A_{11} is independently a basic, a neutral/polar, a hydrophobic or a small amino acid or is proline;

A_{17} is not present or, if present, is a basic, a neutral/polar, a hydrophobic or a small amino acid;
25   A_{18} is not present or, if present, is a basic, a hydrophobic, a neutral/polar or a small amino acid, or a modified form of Formula (1) and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof wherein at least one of the 4 cysteines is
30 independently replaced by a hydrophobic amino acid or a small amino acid;

with the proviso that the compound of Formula (1) must have a charge of +3 or greater.

In still other aspects, the invention is directed to
35 recombinant materials useful for the production of the peptides of the invention as well as plants or animals modified to contain expression systems for the production of these peptides. The invention is also directed to

pharmaceutical compositions and compositions for application
to plants containing the peptides of the invention as active
ingredients or compositions which contain expression systems
for production of the peptides or for *in situ* expression of
5   the nucleotide sequence encoding these peptides. The
invention is also directed to methods to prepare the
invention peptides synthetically, to antibodies specific for
these peptides, and to the use of the peptides as
preservatives.
10   In other aspects, the invention is directed to the use
of the compounds of the invention as standards in
antimicrobial assays. The compounds many also be used as
antimicrobials in solutions useful in eye care, such as
contact lens solutions, and in topical or other
15 pharmaceutical compositions for treatment of sexually
transmitted diseases (STDs). The invention is also directed
to use of the invention compounds as preservatives for foods
or other perishables. As the invention peptides can
inactivate endotoxin, the invention is also directed to a
20 method to inactivate endotoxins using the compounds of the
invention and to treat gram-negative sepsis by taking
advantage of this property.

Brief Description of the Drawings
25   Figure 1 shows the elution pattern of a concentrate of
the ultrafiltrate of porcine leukocytes applied to a Biogel
P10 column.
         Figure 2 shows the antibacterial activity of the P10
fractions obtained from elution of the column described in
30 Figure 1.
         Figure 3 shows an elution pattern obtained when
fractions 76-78 from the Biogel P10 column of Figure 1 is
applied to HPLC.
         Figure 4 shows the antimicrobial activity of the
35 purified porcine protegrins of the invention:
             Figure 4a shows antibacterial activity against *E. Coli*;
             Figure 4b shows antibacterial activity against *Listeria
monocytogenes*;

Figure 4c shows antifungal activity against *Candida albicans*;

Figure 4d shows antibacterial activity against *S. aureus*.

Figure 4e shows antibacterial activity against *K. pneumoneae*.

Figure 5 shows the effect of various test conditions on antimicrobial activity:

Figure 5a shows activity against *Candida albicans* in 100 μM NaCl;

Figure 5b shows activity against *E. Coli* in 100 μM NaCl;

Figure 5c shows activity against *Candida albicans* in 90% fetal calf serum.

Figure 6 shows the antimicrobial activity of the linear forms of the protegrins under various test conditions:

Figure 6a shows the activity against *E. coli* in 10 mM phosphate-citrate buffer, pH 6.5;

Figure 6b shows the activity against *E. coli* in the same buffer with 100 mM NaCl;

Figure 6c shows the activity against *L. monocytogenes* in the buffer of Figures 6a-6b;

Figure 6d shows the activity against *L. monocytogenes* in the same buffer with the addition of 100 mM NaCl;

Figure 6e shows the activity against *C. albicans* in the presence of 10 mM phosphate; and

Figure 6f shows the activity against *C. albicans* in the presence of 10 mM phosphate plus 100 mM NaCl.

Figure 7 shows a composite of cDNA encoding the precursors of PG-1, PG-2, PG-3 and PG-4.

Figure 8 shows the nucleotide sequence and the deduced amino acid sequence of the genomic DNA encoding the precursor protein for the antimicrobial compounds of the invention PG-1, PG-3, and PG-5.

Figure 9 shows the organization of the protegrin genomic DNA.

Figure 10 shows the amino acid sequences of the protegrins PG-1 to PG-5.

Figures 11a-11c show the antimicrobial activity of synthetically prepared PG-5 as compared to that of synthetically prepared PG-1.

Figures 12a-12d show the effects of various protegrins against various target microbes.

Figure 13 shows a graphical representation of the effects of the kite and bullet forms of PG-1 against gram positive bacteria.

Figure 14 shows a graphical representation of the effects of the kite and bullet forms of PG-1 against gram negative bacteria.

Figure 15 is a graphical representation of the antimicrobial activity of the snake form of PG-1 against gram positive bacteria.

Figure 16 is a graphical representation of the antimicrobial activity of the snake form of PG-1 against gram negative bacteria.

Modes of Carrying Out the Invention

The peptides of the invention are described by the formula:

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \quad (1)$$

and its defined modified forms. Those peptides which occur in nature must be in purified and isolated form or prepared recombinantly.

The designation A_n in each case represents an amino acid at the specified position in the peptide. As A_{17} and A_{18} may or may not be present, the peptides of the invention contain either 16, 17 or 18 amino acids. The positions of the cysteine residues, shown as C in Formula (1), are invariant in the peptides of the invention; however, in the modified forms of the peptides of Formula (1), also included within the scope of the invention, at least one of 1-4 of

these cysteines may be replaced by a hydrophobic or small amino acid.

The amino terminus of the peptide may be in the free amino form or may be acylated by a group of the formula
5   RCO-, wherein R represents a hydrocarbyl group of 1-6C. The hydrocarbyl group is saturated or unsaturated and is typically, for example, methyl, ethyl, i-propyl, t-butyl, n-pentyl, cyclohexyl, cyclohexene-2-yl, hexene-3-yl, hexyne-4-yl, and the like.

10   The C-terminus of the peptides of the invention may be in the form of the underivatized carboxyl group, either as the free acid or an acceptable salt, such as the potassium, sodium, calcium, magnesium, or other salt of an inorganic ion or of an organic ion such as caffeine. The carboxyl
15   terminus may also be derivatized by formation of an ester with an alcohol of the formula ROH, or may be amidated by an amine of the formula NH_3, or RNH_2, or R_2NH, wherein each R is independently hydrocarbyl of 1-6C as defined above. Amidated forms of the peptides wherein the C-terminus has
20   the formula $CONH_2$ are preferred.

As the peptides of the invention contain substantial numbers of basic amino acids, the peptides of the invention may be supplied in the form of the acid addition salts. Typical acid addition salts include those of inorganic ions
25   such as chloride, bromide, iodide, fluoride or the like, sulfate, nitrate, or phosphate, or may be salts of organic anions such as acetate, formate, benzoate and the like. The acceptability of each of such salts is dependent on the intended use, as is commonly understood.

30   The peptides of the invention that contain at least two cysteines may be in straight-chain or cyclic form. The straight-chain forms are convertible to the cyclic forms, and vice versa. Methods for forming disulfide bonds to create the cyclic peptides are well known in the art, as are
35   methods to reduce disulfides to form the linear compounds. The linear compounds can be stabilized by addition of a suitable alkylating agent such as iodoacetamide.

The cyclic forms are the result of the formation of cystine linkages among all or some of the four invariant cysteine residues. Cyclic forms of the invention include all possible permutations of cystine bond formation; if the cysteines are numbered in order of their occurrence starting at the N-terminus as C_6, C_8, C_{13} and C_{15}, these permutations include:

C_6-C_8 ;
C_6-C_{13};
C_6-C_{15};
C_8-C_{13};
C_8-C_{15};
C_{13}-C_{15};
C_6-C_8, C_{13}-C_{15};
C_6-C_{13}, C_8-C_{15}; and
C_6-C_{15}, C_8-C_{13}.

In the modified forms of the peptides, where 1-4 cysteines are replaced, similar permutations are available when 2-3 cysteines are present.

The native forms of the protegrins contain two cystine bonds are between the cysteine at position 6 and the cysteine at position 15 and the other between the cysteine at position 8 and the cysteine at position 13. Accordingly, in those embodiments having two cystine linkages, the C_6-C_{15}, C_8-C_{13} form is preferred. However, it has been found by the present applicants that forms of the protegrins containing only one cystine linkage are active and easily prepared. Preferred among embodiments having only one cystine linkage are those represented by C_6-C_{15} alone and by C_8-C_{13} alone.

Forms containing a C_6-C_{15} cystine as the only cystine linkage are generally designated "bullet" forms of the protegrins; those wherein the sole cystine is C_8-C_{13} are designated the "kite" forms. The bullet and kite forms can most conveniently be made by replacing the cystines at the positions not to be linked by cystine with a neutral amino acid, preferably a small amino acid such as glycine, serine, alanine or threonine and less preferably a neutral polar amino acid such as asparagine or glutamine. Thus, in

embodiments of the bullet form, each of C_8 and C_{13} is
independently alanine, serine, threonine or glycine,
preferably both are alanine. Conversely, in the kite form,
C_6 and C_{15} are thus replaced.

5   As the linearalized forms of the native cyclic peptides
have valuable activities, even when chemically stabilized to
preserve the sulfhydryl form of cysteine for example, by
reaction with iodoacetamide, the compounds of the invention
also include linearalized forms which are stabilized with
10   suitable reagents. As defined herein, "SH-stabilized" forms
of the peptides of the invention contain sulfhydryl groups
reacted with standard reagents to prevent reformation into
disulfide linkages.

An alternative approach to providing linear forms of
15   the protegrins of the invention comprises use of the
modified form of the peptides where cysteine residues are
replaced by amino acids which do not form cystine linkages.
In this instance, too, all 4 (or at least 3) of the cystines
at positions 6, 8, 13, and 15 are replaced by polar neutral
20   or small amino acids as listed above. It is preferred that
all 4 cysteine residues be replaced in order to minimize the
likelihood of intermolecular bonding.

The amino acids denoted by A_n may be those encoded by
the gene or analogs thereof, and may also be the D-isomers
25   thereof. One preferred embodiment of the peptides of the
invention is that form wherein all of the residues are in
the D-configuration thus conferring resistance to protease
activity while retaining antimicrobial or antiviral
properties. The resulting protegrins are themselves
30   enantiomers of the native L-amino acid-containing forms.

The amino acid notations used herein are conventional
and are as follows:

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Amino Acid	One-Letter Symbol	Three-Letter Symb l
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

The amino acids not encoded genetically are abbreviated as indicated in the discussion below.

In the specific peptides shown in the present

5   application, the L-form of any amino acid residue having an optical isomer is intended unless the D-form is expressly indicated by a dagger superscript (†).

The compounds of the invention are peptides which are partially defined in terms of amino acid residues of

10   designated classes. Amino acid residues can be generally subclassified into major subclasses as follows:

Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the

15   conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface

positions in the conformation of a peptide in which it is
contained when the peptide is in aqueous medium at
physiological pH.

Hydrophobic: The residues are not charged at
5   physiological pH and the residue is repelled by aqueous
solution so as to seek the inner positions in the
conformation of a peptide in which it is contained when the
peptide is in aqueous medium.

Neutral/polar: The residues are not charged at
10   physiological pH, but the residue is not sufficiently
repelled by aqueous solutions so that it would seek inner
positions in the conformation of a peptide in which it is
contained when the peptide is in aqueous medium.

This description also characterizes certain amino acids
15   as "small" since their side chains are not sufficiently
large, even if polar groups are lacking, to confer
hydrophobicity. "Small" amino acids are those with four
carbons or less when at least one polar group is on the side
chain and three carbons or less when not.

20   It is understood, of course, that in a statistical
collection of individual residue molecules some molecules
will be charged, and some not, and there will be an
attraction for or repulsion from an aqueous medium to a
greater or lesser extent. To fit the definition of
25   "charged," a significant percentage (at least approximately
25%) of the individual molecules are charged at
physiological pH. The degree of attraction or repulsion
required for classification as polar or nonpolar is
arbitrary and, therefore, amino acids specifically
30   contemplated by the invention have been classified as one or
the other. Most amino acids not specifically named can be
classified on the basis of known behavior.

Amino acid residues can be further subclassified as
cyclic or noncyclic, and aromatic or nonaromatic, self-
35   explanatory classifications with respect to the side-chain
substituent groups of the residues, and as small or large.
The residue is considered small if it contains a total of
four carbon atoms or less, inclusive of the carboxyl carbon,

provided an additional polar substituent is present; three
or less if not. Small residues are, of course, always
nonaromatic.

For the naturally occurring protein amino acids,
subclassification according to the foregoing scheme is as
follows.

Acidic: Aspartic acid and Glutamic acid;

Basic: Noncyclic: Arginine, Lysine;
           Cyclic: Histidine;

Small: Glycine, Serine, Alanine, Threonine;

Polar/large: Asparagine, Glutamine;

Hydrophobic: Tyrosine, Valine, Isoleucine, Leucine,
                Methionine, Phenylalanine, Tryptophan.

The gene-encoded secondary amino acid proline is a
special case due to its known effects on the secondary
conformation of peptide chains, and is not, therefore,
included in a group. Cysteine residues are also not
included in these classifications since their capacity to
form disulfide bonds to provide secondary structure is
critical in the compounds of the present invention.

Certain commonly encountered amino acids, which are not
encoded by the genetic code, include, for example, beta-
alanine (beta-Ala), or other omega-amino acids, such as
3-aminopropionic, 2,3-diaminopropionic (2,3-diaP),
4-aminobutyric and so forth, alpha-aminisobutyric acid
(Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit),
t-butylalanine (t-BuA), t-butylglycine (t-BuG),
N-methylisoleucine (N-MeIle), phenylglycine (Phg), and
cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine
(2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
(Tic); β-2-thienylalanine (Thi); methionine sulfoxide (MSO);

and homoarginine (Har). These also fall conveniently into
particular categories.

       Based on the above definitions,

       Sar, beta-Ala, 2,3-diaP and Aib are small;

5   t-BuA, t-BuG, N-MeIle, Nle, Mvl, Cha, Phg, Nal, Thi and
Tic are hydrophobic;

       Orn and Har are basic;

       Cit, Acetyl Lys, and MSO are neutral/polar.

       The various omega-amino acids are classified according
10 to size as small (beta-Ala and 3-aminopropionic) or as large
and hydrophobic (all others).

       Other amino acid substitutions of those encoded in the
gene can also be included in peptide compounds within the
scope of the invention and can be classified within this
15 general scheme according to their structure.

       In all of the peptides of the invention, one or more
amide linkages (-CO-NH-) may optionally be replaced with
another linkage which is an isostere such as $-CH_2NH-$, $-CH_2S-$,
$-CH_2CH_2$, -CH=CH- (cis and trans), $-COCH_2-$, $-CH(OH)CH_2-$ and
20 $-CH_2SO-$. This replacement can be made by methods known in
the art. The following references describe preparation of
peptide analogs which include these alternative-linking
moieties: Spatola, A.F., Vega Data (March 1983), Vol. 1,
Issue 3, "Peptide Backbone Modifications" (general review);
25 Spatola, A.F., in "Chemistry and Biochemistry of Amino Acids
Peptides and Proteins," B. Weinstein, eds., Marcel Dekker,
New York, p. 267 (1983) (general review); Morley, J.S.,
Trends Pharm Sci (1980) pp. 463-468 (general review);
Hudson, D., et al., Int J Pept Prot Res (1979) 14:177-185
30 ($-CH_2NH-$, $-CH_2CH_2-$); Spatola, A.F., et al., Life Sci (1986)
38:1243-1249 ($-CH_2-S$); Hann, M.M., J Chem Soc Perkin Trans I
(1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G., et
al., J Med Chem (1980) 23:1392-1398 ($-COCH_2-$); Jennings-
White, C., et al., Tetrahedron Lett (1982) 23:2533
35 ($-COCH_2-$); Szelke, M., et al., European Application EP 45665
(1982) CA:97:39405 (1982) ($-CH(OH)CH_2-$); Holladay, M.W., et
al., Tetrahedron Lett (1983) 24:4401-4404 ($-C(OH)CH_2-$); and
Hruby, V.J., Life Sci (1982) 31:189-199 ($-CH_2-S-$).

The compounds of Formula (1) are generally defined as

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_7-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \quad (1)$$

and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof, which is either in the optionally -SH stabilized linear or in a cystine-bridged form

wherein A_1 is a basic amino acid;

each of A_2 and A_3 is independently a small amino acid;

each of A_5, A_7, A_{14} is independently a hydrophobic amino acid;

A_4 is a basic or a small amino acid;

each of A_9, A_{12} and A_{16} is independently a basic, a hydrophobic, a neutral/polar or a small amino acid;

each of A_{10} and A_{11} is independently a basic, a neutral/polar, a hydrophobic or a small amino acid or is proline;

A_{17} is not present or, if present, is a basic, a neutral/polar, a hydrophobic or a small amino acid;

A_{18} is not present or, if present, is a basic, a hydrophobic, a neutral/polar or a small amino acid, or a modified form of Formula (1) and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof wherein at least one of the 4 cysteines is independently replaced by a hydrophobic amino acid or a small amino acid;

with the proviso that the compound of Formula (1) must have a charge of +3 or greater.

In preferred embodiments of the compounds of the invention, each of A_1 and A_9 is independently selected from the group consisting of R, K and Har; more preferably, both A_1 and A_9 are R.

In another class of preferred embodiments, each of A_2 and A_3 is independently selected from the group consisting of G, A, S and T; more preferably, A_2 and A_3 are G.

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In another set of preferred embodiments, A_4 is selected from the group consisting of R, K, Har, G, A, S and T; more preferably, A_4 is R or G.

In another set of preferred embodiments, each of A_5, A_{14} and A_{16} is independently selected independently from the group consisting of I, V, L, Nle and F; preferably I, V, L and F.

In another set of preferred embodiments, each of A_7 and A_{12} is independently selected from the group consisting of I, V, L, W, Y and F; preferably A_7 is Y and A_{12} is I or F.

In another set of preferred embodiments, A_{10} is R, G or P.

In another set of preferred embodiments, A_{11} is R or W.

A_{17}, when present, is preferably G, A, S or T, most preferably G;

A_{18}, when present, is preferably R, K or Har, most preferably R.

As described above, the compounds of Formula (1) are either in cyclic or noncyclic (linearalized) form or may be modified wherein 1-4 of the cysteines is replaced by a small amino acid residue or a hydrophobic residue or a nonpolar large amino acid residue. If the linearalized forms of the compound of Formula (1) are prepared, or if linearalized forms of those modified peptides which contain at least two cysteines are prepared, it is preferred that the sulfhydryl groups be stabilized by addition of a suitable reagent. Preferred embodiments for the hydrophobic amino acid to replace cysteine residues are I, V, L and NLe, preferably I, V or L. Preferred small amino acids to replace the cysteine residues include G, A, S and T, most preferably G. Preferred large polar amino acids are N and Q.

In an alternative embodiment, the peptides of the invention are defined as described by Formula (1), but wherein the definitions of A_n in each case are determined by the isolatability of the peptide from animal leukocytes by the invention method. The invention method comprises the steps of providing an ultrafiltrate of a lysate of animal leukocytes and isolating peptides of 16-18 amino acids.

These peptides can further be defined by the ability of DNA encoding them to hybridize under stringent conditions to DNA encoding the peptides exemplified as PG-1, PG-2, PG-3, PG-4 and PG-5 herein.

5   Particularly preferred compounds of the invention are:

Unmodified forms
   PG-1:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R
   PG-2:   R-G-G-R-L-C-Y-C-R-R-R-F-C-I-C-V
10 PG-3:   R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R
   PG-4:   R-G-G-R-L-C-Y-C-R-G-W-I-C-F-C-V-G-R
   PG-5:   R-G-G-R-L-C-Y-C-R-P-R-F-C-V-C-V-G-R
           R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V
           K-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V
15   R-G-G-Har-L-C-Y-C-R-R-R-F-C-V-C-V
           R-G-G-Har-L-C-Y-C-Har-R-R-F-C-V-C-V-G-R
           R-G-G-R-V-C-Y-C-R-Har-R-F-C-V-C-V-G-R
           R-G-G-R-L-C-Y-C-R-K-K-W-C-V-C-V-G-R
           R-G-G-R-L-C-Y-C-R-Har-R-Y-C-V-C-V-G-R
20   R-G-S-G-L-C-Y-C-R-R-K-W-C-V-C-V-G-R
           R-A-T-R-I-C-F-C-R-R-R-F-C-V-C-V-G-R
           R-G-G-K-V-C-Y-C-R-Har-R-F-C-V-C-V-G-R
           R-A-T-R-I-C-F-C-R†-R-R-F-C-V-C-V-G-R†
           R-G-G-K-V-C-Y-C-R-Har†-R-F-C-V-C-V-G-R
25 PG-1:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R (all †)
   PG-2:   R-G-G-R-L-C-Y-C-R-R-R-F-C-I-C-V (all †)
   PG-3:   R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R (all †)
   PG-4:   R-G-G-R-L-C-Y-C-R-G-W-I-C-F-C-V-G-R (all †)
   PG-5:   R-G-G-R-L-C-Y-C-R-P-R-F-C-V-C-V-G-R
30 PC-39: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-R
   PC-41: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G
   PC-100: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-Y
   PC-101: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-T
   PC-102: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-A
35 PC-103: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-L
   PC-104: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-I
   PC-105: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-F

```
      PC-106:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W
      PC-108:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R
               R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R
               R-G-G-R-L-C-W-C-R-R-R-F-C-V-C-V-G-R
  5            R-G-G-R-L-C-Y-C-R-R-R-W-C-V-C-V-G-R
               R-G-G-R-L-C-Y-C-R-R-R-F-C-W-C-V-G-R
               R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W-G-R
      IB-247:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH
      IB-249:  R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH
 10   IB-223:  R-G-G-G-L-C-Y-C-R-R-G-F-C-V-C-F-G-R
      IB-224:  R-G-G-G-L-C-Y-C-R-R-P-F-C-V-C-V-G-R
      IB-324:  R-G-G-G-L-C-Y-C-R-P-R-F-C-V-C-V-G-R-OH
      IB-341:  R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R-OH (X=NMeG)
      IB-342:  R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R (X=NMeG)
 15   IB-384:  R-G-G-R-L-C-Y-C-X-G-R-F-C-V-C-V-G-R (X=Cit)
      IB-398:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R
      IB-399:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R-OH
      IB-218:  R-G-G-G-L-C-Y-C-F-P-K-F-C-V-C-V-G-R
      IB-349:  R-G-G-R-L-C-Y-C-R-X-R-Cha-C-V-C-W-G-R (X=NMeG)
 20   IB-350:  R-G-G-R-W-C-V-C-R-X-R-Cha-C-Y-C-V-G-R (X=NMeG)
      IB-394:  R-G-G-R-W-C-V-C-R-G-R-Cha-C-Y-C-V-G-R
      IB-416:  R-G-G-R-L-C-Y-C-R-R-R-F-C-NMeV-C-V-G-R
      IB-400:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V
      IB-401:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-OH
 25
```

both the linear and mono- and bicyclic forms thereof, and
including the N-terminal acylated and C-terminal amidated
forms;

30 **Modified forms**

```
               R-G-G-R-L-V-Y-C-R-R-R-F-C-V-C-V-G-R
               R-G-G-R-L-G-Y-C-R-R-R-F-C-I-C-V
               R-G-G-G-L-C-Y-G-R-R-R-F-C-V-C-V-G-R
               R-G-G-R-L-G-Y-G-R-R-R-F-G-V-C-V
 35            K-G-G-R-L-V-Y-V-R-R-R-F-I-V-C-V
               R-G-G-Har-L-C-Y-C-R-R-R-F-C-V-G-V
               R-G-G-Har-L-C-Y-C-Har-R-R-F-C-V-L-V-G-R
               R-G-G-R-V-C-Y-V-R-Har-R-F-L-V-G-V-G-R
```
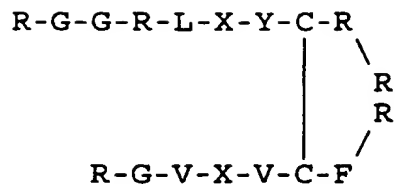
```
          R-G-G-R-L-C-Y-S-R-K-K-W-C-V-S-V-G-R
          R-G-G-R-L-C-Y-C-R-Har-R-Y-S-V-V-V-G-R
          R-G-S-G-L-S-Y-C-R-R-K-W-G-V-C-V-G-R
          R-A-T-R-I-S-F-S-R-R-R-F-S-V-S-V-G-R
   5      R-G-G-K-V-C-Y-G-R-Har-R-F-S-V-C-V-G-R
          R-A-T-R-I-V-F-C-R†-R-R-F-G-V-C-V-G-R†
          R-G-G-K-V-C-Y-L-R-Har†-R-F-L-V-C-V-G-R
          R-G-G-R-I-C-F-L-R-P-R-I-G-V-C-V-G-R
   PC-49:  R-G-G-R-L-C-W-A-R-R-R-F-A-V-C-V-G-R
  10  PC-50:  R-G-G-R-L-C-Y-A-R-R-R-W-A-V-C-V-G-R
      PC-52:  R-G-G-R-L-A-W-C-R-R-R-F-C-V-A-V-G-R
      PC-53:  R-G-G-R-L-A-Y-C-R-R-R-F-C-V-A-W-G-R
      PC-55:  R-G-G-R-L-A-W-A-R-R-R-F-A-V-A-V-G-R
      PC-56:  R-G-G-R-L-A-Y-A-R-R-R-W-A-V-A-V-G-R
  15  PC-57:  R-G-G-R-L-A-Y-A-R-R-R-F-A-V-A-W-G-R
      IB-214:  R-G-G-G-L-C-Y-A-R-G-W-I-A-F-C-V-G-R
      IB-216:  R-G-G-G-L-C-Y-A-R-G-F-I-A-V-C-F-G-R
      IB-225:  R-G-G-G-L-C-Y-A-R-P-R-F-A-V-C-V-G-R
      IB-226:  R-G-G-G-L-C-Y-T-R-P-R-F-T-V-C-V-G-R
  20  IB-227:  R-G-G-G-L-C-Y-A-R-K-G-F-A-V-C-V-G-R
      IB-288:  R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R-OH
      IB-289:  R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R
```

both the linear and cyclic (where possible) forms thereof,
25 and including the N-terminal acylated and C-terminal
amidated forms.

        Particularly preferred are compounds wherein a single
cystine bond is formed between C_6 and C_{15} or between C_8 and
C_{13} wherein four compounds having a cystine bond between C_8
30 and C_{13} each of C_6 and C_{15} is independently replaced by "X"
wherein X is a hydrophobic, a small, or a large polar amino
acid. Similarly, where the single cystine bond is between
C_8 and C_{13}, each of C_6 and C_{15} is independently replaced by X
as defined above. Also preferred are the "snake" forms of
35 the compounds of the invention where all 4 cysteines are
replaced by X as defined above. Particularly preferred
embodiments of these compounds of the invention include:

Kite form-1

5
```
        R-G-G-R-L-X-Y-C-R
                        \
                         R
                         R
                        /
        R-G-V-X-V-C-F
```

10   Kite form-2

```
        R-G-G-R-L-X-Y-C-R
                        \
                         R
                         R
                        /
        R-G-V-X-I-C-F
```

15

Kite form-3

20
```
        R-G-G-G-L-X-Y-C-R
                        \
                         R
                         R
                        /
        R-G-V-X-V-C-F
```

25   Kite form-4

```
        R-G-G-R-L-X-Y-C-R
                        \
                         W
                         G
                        /
        R-G-V-X-F-C-I
```

30

Kite form-5

35
```
        R-G-G-R-L-X-Y-C-R
                        \
                         P
                         R
                        /
        R-G-V-X-V-C-F
```

40

Bullet form-1

45
```
        R-G-G-R-L-C-Y-X-R
                        \
                         R
                         R
                        /
        R-G-V-C-V-X-F
```

Bullet form-2

```
          R-G-G-R-L-C-Y-X-R
                  |          \
                  |           R
                  |           R
                  |          /
          R-G-V-C-I-X-F
```

Bullet form-3

```
          R-G-G-G-L-C-Y-X-R
                  |          \
                  |           R
                  |           R
                  |          /
          R-G-V-C-V-X-F
```

Bullet form-4

```
          R-G-G-R-L-C-Y-X-R
                  |          \
                  |           W
                  |           G
                  |          /
          R-G-V-C-F-X-I
```

Bullet form-5

```
          R-G-G-R-L-C-Y-X-R
                  |          \
                  |           P
                  |           R
                  |          /
          R-G-V-C-V-X-F
```

Snake form-1: R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

Snake form-2: R-G-G-R-L-X-Y-X-R-R-R-F-X-I-X-V

Snake form-3: R-G-G-G-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

Snake form-4: R-G-G-R-X-L-X-Y-R-G-W-I-X-F-X-V-G-R

Snake form-5: R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

wherein X is as defined above.

Particularly preferred embodiments of X are those wherein X is a small amino acid, especially S and A, especially A.

Preparation of the Invention Compounds

The invention compounds, often designated herein "protegrins" are essentially peptide backbones which may be modified at the N- or C-terminus and also may contain one or

two cystine disulfide linkages. The peptides may first be
synthesized in noncyclized form. These peptides may then be
converted to the cyclic peptides if desired by standard
methods of cystine bond formation. As applied to the
5   protegrins herein, "cyclic forms" refers to those forms
which contain cyclic portions by virtue of the formation of
disulfide linkages between cysteine residues in the peptide.
If the straight-chain forms are preferred, it is preferable
to stabilize the sulfhydryl groups for any peptides of the
10 invention which contain two or more cysteine residues.

        Standard methods of synthesis of peptides the size of
protegrins are known. Most commonly used currently are
solid phase synthesis techniques; indeed, automated
equipment for systematically constructing peptide chains can
15 be purchased. Solution phase synthesis can also be used but
is considerably less convenient. When synthesized using
these standard techniques, amino acids not encoded by the
gene and D-enantiomers can be employed in the synthesis.
Thus, one very practical way to obtain the compounds of the
20 invention is to employ these standard chemical synthesis
techniques.

        In addition to providing the peptide backbone, the N-
and/or C-terminus can be derivatized, again using
conventional chemical techniques. The compounds of the
25 invention may optionally contain an acyl group, preferably
an acetyl group at the amino terminus. Methods for
acetylating or, more generally, acylating, the free amino
group at the N-terminus are generally known in the art; in
addition, the N-terminal amino acid may be supplied in the
30 synthesis in acylated form.

        At the carboxy terminus, the carboxyl group may, of
course, be present in the form of a salt; in the case of
pharmaceutical compositions this will be a pharmaceutically
acceptable salt. Suitable salts include those formed with
35 inorganic ions such as NH_4^+, Na^+, K^+, Mg^{++}, Ca^{++}, and the like
as well as salts formed with organic cations such as those
of caffeine and other highly substituted amines. The
carboxy terminus may also be esterified using alcohols of

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the formula ROH wherein R is hydrocarbyl (1-6C) as defined above. Similarly, the carboxy terminus may be amidated so as to have the formula $-CONH_2$, -CONHR, or $-CONR_2$, wherein each R is independently hydrocarbyl (1-6C) as herein

5   defined. Techniques for esterification and amidation as well as neutralizing in the presence of base to form salts are all standard organic chemical techniques.

        If the peptides of the invention are prepared under physiological conditions, the side-chain amino groups of the

10   basic amino acids will be in the form of the relevant acid addition salts.

        Formation of disulfide linkages, if desired, is conducted in the presence of mild oxidizing agents. Chemical oxidizing agents may be used, or the compounds may

15   simply be exposed to the oxygen of the air to effect these linkages. Various methods are known in the art. Processes useful for disulfide bond formation have been described by Tam, J.P. et al., Synthesis (1979) 955-957; Stewart, J.M. et al, "Solid Phase Peptide Synthesis" 2d Ed. Pierce Chemical

20   Company Rockford, IL (1984); Ahmed A.K. et al., J Biol Chem (1975) 250:8477-8482 and Pennington M.W. et al., Peptides 1990, E. Giralt et al., ESCOM Leiden, The Netherlands (1991) 164-166. An additional alternative is described by Kamber, B. et al., Helv Chim Acta (1980) 63:899-915. A method

25   conducted on solid supports is described by Albericio Int J Pept Protein Res (1985) 26:92-97.

        A particularly preferred method is solution oxidation using molecular oxygen. This method has been used by the inventors herein to refold synthetic PG-1, PG-3 in its amide

30   or acid forms, *enantio*PG-1 and the two unisulfide PG-1 compounds (C_6-C_{15} and C_8-C_{13}). Recoveries are as high as 30%.

        If the peptide backbone is comprised entirely of gene-encoded amino acids, or if some portion of it is so

35   composed, the peptide or the relevant portion may also be synthesized using recombinant DNA techniques. The DNA encoding the peptides of the invention may itself be synthesized using commercially available equipment; codon

choice can be integrated into the synthesis depending on the
nature of the host. Alternatively, although less
convenient, the DNA can be obtained, at least initially, by
screening a cDNA library prepared from porcine leukocytes
5   using probes or PCR primers based on the sequences of the
protegrins described herein. This results in recovery of
the naturally occurring sequence encoding the protegrins of
the invention. Obtention of this native sequence is
significant for purposes other than the synthesis of the
10   protegrins per se; the availability of the naturally
occurring sequences provides a useful probe to obtain
corresponding DNA encoding protegrins of other species.
Thus, cDNA libraries, for example, of leukocytes derived
from other animals can be screened using the native DNA,
15   preferably under conditions of high stringency. High
stringency is as defined by Maniatis, *et al.* Molecular
Cloning: a Laboratory Manual 2nd Ed, Cold Spring Harbor
Laboratory Press (1989), the relevant portions of which are
incorporated herein by reference. This procedure also
20   permits recovery of allelic variants of these peptides from
the same species.

    Alternatively, the protegrins can be prepared by
isolation from leukocytes of a desired species using
techniques similar to those disclosed herein for the
25   isolation of porcine protegrins. In general, these
techniques involve preparing a lysate of a leukocyte
preparation, ultrafiltering the supernatant of the clarified
lysate and recovering the ultrafiltrate. The ultrafiltrate
is then subjected to chromatographic separation. The
30   location of fragments having antimicrobial and antiviral
activity corresponding to protegrins can be assessed using
criteria of molecular weight and assaying the fractions for
the desired activities as described herein. The native
forms of these peptides are believed to be the cyclic forms;
35   if desired, the linearalized forms can be prepared by
treating the peptides with reducing agents and stabilizing
the sulfhydryl groups that result.

Isolated and recombinantly produced forms of the
protegrins may require subsequent derivatization to modify
the N- and/or C-terminus and, depending on the isolation
procedure, to effect the formation of cystine bonds as
5   described hereinabove. Depending on the host organism used
for recombinant production and the animal source from which
the protein is isolated, some or all of these conversions
may already have been effected.

For recombinant production, the DNA encoding the
10   protegrins of the invention is included in an expression
system which places these coding sequences under control of
a suitable promoter and other control sequences compatible
with an intended host cell. Types of host cells available
span almost the entire range of the plant and animal
15   kingdoms. Thus, the protegrins of the invention could be
produced in bacteria or yeast (to the extent that they can
be produced in a nontoxic or refractile form or utilize
resistant strains) as well as in animal cells, insect cells
and plant cells. Indeed, modified plant cells can be used
20   to regenerate plants containing the relevant expression
systems so that the resulting transgenic plant is capable of
self protection vis-à-vis these infective agents.

The protegrins of the invention can be produced in a
form that will result in their secretion from the host cell
25   by fusing to the DNA encoding the protegrin, a DNA encoding
a suitable signal peptide, or may be produced
intracellularly. They may also be produced as fusion
proteins with additional amino acid sequence which may or
may not need to be subsequently removed prior to the use of
30   these compounds as antimicrobials or antivirals.

Thus, the protegrins of the invention can be produced
in a variety of modalities including chemical synthesis,
recombinant production, isolation from natural sources, or
some combination of these techniques.

35   Those members of the protegrin class which occur
naturally are supplied in purified and isolated form. By
"purified and isolated" is meant free from the environment
in which the peptide normally occurs (in the case of such

naturally occurring peptides) and in a form where it can be
used practically. Thus, "purified and isolated" form means
that the peptide is substantially pure, i.e., more than 90%
pure, preferably more than 95% pure and more preferably more
5   than 99% pure or is in a completely different context such
as that of a pharmaceutical preparation.

Antibodies
        Antibodies to the protegrins of the invention may also
10   be produced using standard immunological techniques for
production of polyclonal antisera and, if desired,
immortalizing the antibody-producing cells of the immunized
host for sources of monoclonal antibody production.
Techniques for producing antibodies to any substance of
15   interest are well known. It may be necessary to enhance the
immunogenicity of the substance, particularly as here, where
the material is only a short peptide, by coupling the hapten
to a carrier. Suitable carriers for this purpose include
substances which do not themselves produce an immune
20   response in the mammal to be administered the hapten-carrier
conjugate. Common carriers used include keyhole limpet
hemocyanin (KLH), diphtheria toxoid, serum albumin, and the
viral coat protein of rotavirus, VP6. Coupling of the
hapten to the carrier is effected by standard techniques
25   such as contacting the carrier with the peptide in the
presence of a dehydrating agent such as
dicyclohexylcarbodiimide or through the use of linkers such
as those available through Pierce Chemical Company, Chicago,
IL.
30   The protegrins of the invention in immunogenic form are
then injected into a suitable mammalian host and antibody
titers in the serum are monitored. It should be noted,
however, that some forms of the protegrins require
modification before they are able to raise antibodies, due
35   to their resistance to antigen processing. For example, the
native form of PG-1, containing two cystine bridges is
nonimmunogenic when administered without coupling to a
larger carrier and was a poor immunogen even in the presence

of potent adjuvants and when coupled through glutaraldehyde
or to KLH. Applicants believe this to be due to its
resistance to attack by leukocyte serine proteases (human
PMN elastase and cathepsin G) as well as to attack by an
5   aspartic protease (pepsin) that resembles several macrophage
cathepsins. The lack of immunogenicity may therefore result
from resistance to processing to a linear form that can fit
in the antigen-presenting pocket of the presenting cell.
Immunogenecity of these forms of the protegrins can be
10   enhanced by cleaving the disulfide bonds.

        Polyclonal antisera may be harvested when titers are
sufficiently high. Alternatively, antibody-producing cells
of the host such as spleen cells or peripheral blood
lymphocytes may be harvested and immortalized. The
15   immortalized cells are then cloned as individual colonies
and screened for the production of the desired monoclonal
antibodies.

        The antibodies of the invention are, of course, useful
in immunoassays for determining the amount or presence of
20   the protegrins. Such assays are essential in quality
controlled production of compositions containing the
protegrins of the invention. In addition, the antibodies
can be used to assess the efficacy of recombinant production
of the protegrins, as well as screening expression libraries
25   for the presence of protegrin encoding genes.

<u>Compositions Containing the Protegrins and Methods of Use</u>
        The protegrins of the invention are effective in
inactivating a wide range of microbial and viral targets,
30   including gram-positive and gram-negative bacteria, yeast,
protozoa and certain strains of virus. Accordingly, they
can be used in disinfectant compositions and as
preservatives for materials such as foodstuffs, cosmetics,
medicaments, or other materials containing nutrients for
35   organisms. For use in such contexts, the protegrins are
supplied either as a single protegrin, in admixture with
several other protegrins, or in admixture with additional
antimicrobial agents. In general, as these are

preservatives in this context, they are usually present in relatively low amounts, of less than 5%, by weight of the total composition, more preferably less than 1%, still more preferably less than 0.1%.

5   The peptides of the invention are also useful as standards in antimicrobial assays and in assays for determination of capability of test compounds to bind to endotoxins such as lipopolysaccharides.

        For use as antimicrobials or antivirals for treatment
10 of animal subjects, the protegrins of the invention can be formulated as pharmaceutical or veterinary compositions. Depending on the subject to be treated, the mode of administration, and the type of treatment desired -- e.g., prevention, prophylaxis, therapy; the protegrins are
15 formulated in ways consonant with these parameters. A summary of such techniques is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA.

        The protegrins are particularly attractive as an active
20 ingredients pharmaceutical compositions useful in treatment of sexually transmitted diseases, including those caused by Chlamydia trachomatis, Treponema pallidum, Neisseria gonorrhoeae, Trichomonas vaginalis, Herpes simplex type 2 and HIV. Topical formulations are preferred and include
25 creams, salves, oils, powders, gels and the like. Suitable topical excipient are well known in the art and can be adapted for particular uses by those of ordinary skill.

        In general, for use in treatment or prophylaxis of STDs, the protegrins of the invention may be used alone or
30 in combination with other antibiotics such as erythromycin, tetracycline, macrolides, for example azithromycin and the cephalosporins. Depending on the mode of administration, the protegrins will be formulated into suitable compositions to permit facile delivery to the affected areas. The
35 protegrins may be used in forms containing one or two disulfide bridges or may be in linear form. In addition, use of the enantiomeric forms containing all D-amino acids may conf r advantages such as resistance to those proteases,

such as trypsin and chymotrypsin, to which the protegrins
containing L-amino acids are less resistant.

      The protegrins of the invention can be administered
singly or as mixtures of several protegrins or in
5   combination with other pharmaceutically active components.
The formulations may be prepared in a manner suitable for
systemic administration or topical or local administration.
Systemic formulations include those designed for injection
(e.g., intramuscular, intravenous or subcutaneous injection)
10 or may be prepared for transdermal, transmucosal, or oral
administration. The formulation will generally include a
diluent as well as, in some cases, adjuvants, buffers,
preservatives and the like. The protegrins can be
administered also in liposomal compositions or as
15 microemulsions.

      If administration is to be oral, the protegrins of the
invention must be protected from degradation in the stomach
using a suitable enteric coating. This may be avoided to
some extent by utilizing amino acids in the D-configuration,
20 thus providing resistance to protease. However, the peptide
is still susceptible to hydrolysis due to the acidic
conditions of the stomach; thus, some degree of enteric
coating may still be required.

      As described in the examples below, the peptides of the
25 invention retain their activity against microbes in the
context of borate solutions that are commonly used in eye
care products. It has also been shown that when tested for
antimicrobial activity against *E. coli* in the presence and
absence of lysozyme in borate buffered saline, that the
30 presence of lysozyme enhanced the effectiveness of PG-3.
This effect was more pronounced when the PG-3 was autoclaved
and similar patterns were obtained for both the free-acid
form and the amide. Accordingly, the protegrins may be used
as preservatives in such compositions or as antimicrobials
35 for treatment of eye infections.

      It is particularly important that the protegrins retain
their activity under physiological conditions including
relatively high saline and in the presence of serum. In

addition, the protegrins are not cytotoxic with respect to
the cells of higher organisms. These properties, described
herein below in the Examples, make them particularly
suitable for *in vivo* and therapeutic use.

5   The protegrins of the invention may also be applied to
plants or to their environment to prevent viral- and
microbial-induced diseases in these plants. Suitable
compositions for this use will typically contain a diluent
as well as a spreading agent or other ancillary agreements
10   beneficial to the plant or to the environment.

Thus, the protegrins of the invention may be used in
any context wherein an antimicrobial and/or antiviral action
is required. This use may be an entirely *in vitro* use, or
the peptides may be administered to organisms.

15   In addition, the antimicrobial or antiviral activity
may be generated *in situ* by administering an expression
system suitable for the production of the protegrins of the
invention. Such expression systems can be supplied to plant
and animal subjects using known techniques. For example, in
20   animals, pox-based expression vectors can be used to
generate the peptides *in situ*. Similarly, plant cells can
be transformed with expression vectors and then regenerated
into whole plants which are capable of their own production
of the peptides.

25   A particularly useful property of the protegrins is
their activity in the presence of serum. Unlike defensins,
protegrins are capable of exerting their antimicrobial
effects in the presence of serum.

As shown hereinbelow, the protegrins are capable of
30   inactivating endotoxins derived from gram-negative
bacteria -- i.e., lipopolysaccharides (LPS) -- in standard
assays. Accordingly, the protegrins may be used under any
circumstances where inactivation of LPS is desired. One
such situation is in the treatment or amelioration of gram-
35   negative sepsis.

The protegrins of the invention, therefore, represent a
peculiarly useful class of compounds because of the
following properties:

1)   they have an antimicrobial effect with respect to a
broad spectrum of target microbial systems, including
viruses, including retroviruses, bacteria, fungi, yeast and
protozoa.

5   2)   Their antimicrobial activity is effective under
physiological conditions - i.e., physiological saline and in
the presence of serum.

3)   They are not toxic to the cells of higher
organisms.

10   4)   They can be prepared in nonimmunogenic form thus
extending the number of species to which they can be
administered.

5)   They can be prepared in forms which are resistant
to certain proteases suggesting they are antimicrobial even
15 in lysosomes.

6) They can be prepared in forms that resist
degradation when autoclaved, thus simplifying their
preparation as components of pharmaceuticals.

The following examples are intended to illustrate but
20 not to limit the invention.

Example 1

Isolation of PG-1, PG-2 and PG-3

Fresh porcine blood was collected into 15-liter vessels
25 containing 5% EDTA in normal saline, pH 7.4 as an
anticoagulant (33 ml/liter blood). The blood cells were
allowed to sediment for 90 minutes at room temperature and
the leukocyte-rich supernatant was removed and centrifuged
at 200 x g for 5.7 minutes. The pellets were pooled and
30 suspended in 0.84% ammonium chloride to lyse erythrocytes
and the resulting leukocytes (70-75% PMN, 5-10% eosinophils,
15-25% lymphocytes and monocytes) were washed in normal
saline, resuspended in ice-cold 10% acetic acid at 10^8/ml,
homogenized and stirred overnight at 4°C. The preparation
35 was centrifuged at 25,000 x g for 3 hours at 4°C and the
supernatant was lyophilized and weighed.

950 mg (dry weight) of lyophilized extract, which
contained 520 mg protein by BCA analysis, was stirred
overnight at 4°C in 100 ml of 10% acetic acid and then
centrifuged at 25,000 x g for 2 hours. The supernate was
5   removed and passed by pressure through a 50 ml stirred
ultracentrifugation cell (Amicon, Danvers, MA) that
contained a YM-5 filter. The ultrafiltrate (24.5 mg protein
by BCA) was concentrated to 3 ml by vacuum centrifugation
(SpeedVac Concentrator, Savant Instruments, Hicksville, NY),
10 applied to a 2.5 x 117 cm BioGel P10 column (Bio-Rad,
Hercules, CA) and eluted at 4°C with 5% acetic acid.

Fractions containing 6.6 ml were obtained. Fractions
were assayed by absorption at 280 nm and the elution pattern
is shown in Figure 1.

15 Aliquots (66 µl) of each fraction were dried by vacuum
centrifugation and resuspended in 6.6 µl of 0.01% acetic
acid. Five µl samples of this concentrate were tested for
antimicrobial activity against *E. coli* ML-35,
L. monocytogenes, strain EGD and *C. albicans*, strain 820,
20 using radiodiffusion and gel overlay techniques as described
by Lehrer, R.I. *et al.* J Immunol Meth (1991) 137:167-173.
Briefly, the underlay agars used for all organisms had a
final pH of 6.5 and contained 9 mM sodium phosphate/1 mM
sodium citrate buffer, 1% w/v agarose and 0.30 µg/ml
25 tryptocase soy broth powder (BBL Cockeysville, MD). The
units of activity in the radial diffusion assay were
measured as described; 10 units correspond to a 1 mm
diameter clear zone around the sample well. 'Activities
obtained for the various fractions are shown in Figure 2.
30 Activity was found in a large number of fractions.

The active fractions were further examined by acid-urea
PAGE (AU-PAGE) and SDS PAGE. Results of these analyses
showed that active antimicrobial peptides of the appropriate
molecular weight were present and concentrated in fractions
35 76-78.

Fractions 76-78 from the Biogel P10 column were then
pooled and chromatographed on a 1 x 25 cm Vydac 218 TP1010

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column with a gradient (buffer A is 0.1% TFA; buffer B is
0.1% TFA in acetonitrile) the increase in acetonitrile
concentration was 1% per minute. The results, assessed in
terms of absorbance at 280 nm and at 225 nm are shown in

5   Figure 3. The peaks corresponding the three peptides
illustrated herein are labeled in the figure. The figure
also contains an inset which shows the results of an acid-
urea PAGE gel stained with Comassie Blue that contains a
starting mixture composed of the pooled fractions and the

10 individual PG species. These are labeled M, 1, 2 and 3 on
the inset. The results clearly show the presence of three
distinct proteins.

The isolated proteins were subjected to amino acid
analysis using three independent methods, and to Edman

15 degradation, chymotrypsin digestion, and fast atom
bombardment mass spectrometric analysis. The peptides,
named "protegrins", are shown to have the amino acid
sequences as follows:

PG-1:   RGGRLCYCRRRFCVCVGR

20 PG-2:   RGGRLCYCRRRFCICV

PG-3:   RGGGLCYCRRRFCVCVGR,

and are amidated at the C-terminus.

The amidation status of the isolated peptides was
established by synthesis of PG-3 both in the free carboxyl

25 and carboxyamidated forms. These synthetic peptides were
then compared to isolated PG-3 using AU-PAGE and also using
reverse-phase HPLC. In both cases, the native product
comigrated with the synthetic amidated form.

The location of the disulfide linkages in the isolated

30 protegrins was also studied using PG-2 as a model. The
determination was performed using sequential enzyme
digestion (chymotrypsin followed by thermolysin) with direct
analysis using LC-ESI-MS on the fragments obtained. The
results of these analyses showed that the two intramolecular

35 disulfide bonds were C_6-C_{15} and C_8-C_{13}. With the location of
the disulfides in these positions, the protegrin molecules

are likely to exist as anti-parallel β sheets similar to the
tachyplesins in overall conformation.

The antimicrobial proteins above are present in much
lower concentrations in initial extracts than are the rabbit
5   defensins in corresponding crude extracts where the
defensins constitute more than 15% of the total protein in
rabbit granulocytes. Using the AU-PAGE analytical method on
the various stages of purification, the peptides are only
faintly visible in the crude extracts, whereas corresponding
10   crude extracts of rabbit granulocytes clearly show the
presence of the defensins. The peptides of the invention
become clearly evident only after the ultrafiltration step.

Because the protegrins whose structures are set forth
above show sequence homology to the decapeptide region
15   corresponding to residues 1-10 of rabbit defensin NP-3a in
the decapeptide region at positions 4-13 of PG-3, the
protegrins, and in particular PG-3, may share the property
of defensin NP-3a in being capable of competitively
antagonizing ACTH-mediated steroid synthesis by adrenocytes.
20   This property, called "corticostasis", may influence the
effectiveness of the protegrins as antiinfectious agents
when employed *in vivo*.

Example 2

25   Antimicrobial Activity

The radial diffusion assay in agarose gels described in
Example 1 was also used to test the activity of the purified
protegrins. Figures 4a, 4b and 4c show the results against
three test organisms in units described as above. The
30   rabbit defensin (NP-1) and the human defensin (HNP-1) were
used as controls.

Figure 4a shows that PG-1 and PG-3 are more effective
against *E. coli* ML-35P than HNP-1 and only slightly less
effective than NP-1. PG-1 and PH-3 were also effective
35   against *Listeria monocytogenes*, strain EGD as shown in
Figure 4b. In Figure 4c, PG-1 and PG-3 were also shown
effective against *Candida albicans*. In general, these

peptides are approximately as effective as rabbit defensin
NP-1 on a weight basis and are more effective than HNP-1.
In all cases, PG-2 was also effective against the three
organisms tested but was not as active as the other two
5   peptides.

In addition to its activity in inhibiting the growth of
the above-mentioned organisms, the PG-1 of the invention has
been shown directly to inhibit the growth of *Staphylococcus
aureus* (see Figure 4d) and *K. pneumoneae* 270 (Figure 4e).
10   HNP-1 used as a control was less effective against *S. aureus*
and almost entirely ineffective against *K. pneumoneae*.

The protegrins of the invention have also been tested
against various other organisms and show broad spectrum
activity.   In addition to their effectiveness in inhibiting
15   the growth of or infection by microorganisms associated with
STDs as described in Example 9 hereinbelow, the protegrins
show strong activity against the following microorganisms in
addition to those tested hereinabove: *Pseudomonas
aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium,*
20   *Staphylococcus aureus, Histoplasma capsulatum, Myobacterium
avium-intracellulare,* and *Mycobacterium tuberculosis.*   The
protegrins showed only fair activity against *Vibrio
vulnificus* and were inactive against *Vibrio cholerae* and
Borrelia burgdorferi.
25

Example 3
Retention of Activity Under Certain Conditions
The antimicrobial activity of the invention compounds
was tested as set forth above, but under conditions of 100μM
30   NaCl and in the presence of 90% fetal calf serum. Figures
5a and 5b show that PG-1 and PG-3 retain their activity with
respect to *C. albicans* and *E. coli* respectively, even in the
presence of 100mM NaCl. Neither NP-1 nor HNP-1 have this
property. Figure 5c shows that although NP-1 and NHP-2 lose
35   their ability to inactivate *C. albicans* in 90% fetal calf
serum, inactivation by PG-3 is retained.

      Accordingly, the protegrins of the invention retain their antimicrobial properties under useful physiological conditions, including isotonic and borate solutions appropriate for use in eye care products.

5   In addition, synthetic PG-1 was tested with respect to its activity against *E. coli* ML-35 (serum sensitive) in underlayered gels containing only 10 mM sodium phosphate buffer, pH 7.4 and a 1:100 dilution of trypticase soy broth, both in the presence and absence of 2.5% normal human serum,

10 which is below the lytic concentration for this strain of *E. coli*. In the presence of serum, the minimal bacteriocidal concentration was reduced from approximately 1.0 µg/ml to about 0.1 µg/ml. This type of effect was not observed either for a linear fragment of cathepsin G or for the

15 defensin HNP-1.

      Similarly, using *C. albicans* as a target organism, underlayers were prepared with 10 mM sodium phosphate with and without 10% normal human serum. The minimal fungicidal concentration fell from about 1.3 µg/ml in the absence of

20 serum to 0.14 µg/ml in its presence. The serum itself at this concentration did not effect *C. albicans*.

      Thus, not only is the action of the protegrins not inhibited by the presence of serum, it is enhanced thereby. Similar results were obtained using *L. monocytogenes* as the

25 target organism.

      The protegrins PG-1 and PG-3 were incubated for 4 hours at pH 2.0 with 0.5 µg/ml pepsin and then neutralized. The residual antimicrobial activity against *C. albicans*, *E. coli* and *L. monocytogenes* was assessed and found to be fully

30 retained. Similar experiments show that these compounds are not degraded by human leukocyte elastase or by human leukocyte cathepsin G even when exposed to high concentrations of these enzymes and at a pH of 7.0 - 8.0 favorable for proteolytic activity. In addition, synthetic

35 PG-3 amide and synthetic PG-3 acid were autoclaved and tested for antimicrobial activity against *E. coli*, *L. monocyogenese* and *C. albicans*; retaining full antimicrobial

activity in all cases.   It is possible that the stability of
these compounds to protease degradation and to autoclaving
is enhanced by the presence of disulfide bonds.

5   Example 4
                      Ability to Bind Endotoxin
            The protegrins of the invention were tested for their
ability to bind the lipid polysaccharide (LPS) of the gram-
negative bacterium *E. coli* strain 0.55B5.   The assay was the
10   *Limulus* amebocyte lysate (LAL) test for endotoxins conducted
in the presence and absence of the test compounds.   The test
was conducted using the procedure described in Sigma
Technical Bulletin No. 210 as revised in December 1992 and
published by Sigma Chemical Company, St. Louis, MO.
15   The LAL test is based on the ability of LPS to effect
gelation in the commercial reagent E-Toxate™ which is
prepared from the lysate of circulating amebocytes of the
Horseshoe Crab *Limulus polyphemus*.   As described in the
technical bulletin, when exposed to minute quantities of
20   LPS, the lysate increases in opacity as well as viscosity
and may gel depending on the concentration of endotoxin.
The technical bulletin goes on to speculate that the
mechanism appears analogous to the clotting of mammalian
blood and involves the steps of activation of a trypsin-like
25   preclotting enzymes by the LPS in the presence of calcium
ion, followed by enzymic modifications of a "coagulogen" by
proteolysis to produce a clottable protein.   These steps are
believed tied to the biologically active or "pyrogenic"
portion of the molecule.   It has been shown previously that
30   detoxified LPS (or endotoxin) gives a negative LAL test.
            The test compounds were used at various concentrations
from 0.25 µg-10 µg in a final volume of 0.2 ml and the test
mixtures contained LPS at a final concentration of 0.05
endotoxin unit/ml and E-Toxate™ at the same concentration.
35   The test compounds were incubated together with the LPS for
15 minutes before the E-Toxate™ was added to a final volume

after E-Toxate™ addition of 0.2 ml. The tubes were then incubated for 30 minutes at 37°C and examined for the formation of a gel.

Both isolated native protegrins (nPGs) and
5 synthetically prepared protegrins (sPGs) were tested. The sPGs were prepared with a carboxyl group at the C-terminus or with an amidated C-terminus. The nPGs are amidated at the C-terminus. Also tested were six different rabbit defensins (NPs) and four native human defensins (HNPs). The
10 results are shown in Table 1.

Table 1						
Peptide	**10 µg**	**5 µg**	**2.5 µg**	**1.0 µg**	**0.5 µg**	**0.25 µg**
nPG-1	no gel	no gel	no gel	no gel	+	+ +
nPG-2	no gel	no gel	no gel	no gel	+	+ +
nPG-3	no gel	no gel	trace	+ +	+ +	+ +
sPG-3 acid	no gel	no gel	trace	+ +	+ +	+ +
sPG-3 amide	no gel	no gel	no gel	+	+ +	+ +
NP-1	not tested	not tested	+ +	+ +	+ +	+ +
NP-2	trace	+	+	+ +	+ +	+ +
NP-3a	no gel	no gel	no gel	+ +	+ +	+ +
NP-3b	no gel	no gel	+	+ +	+ +	+ +
NP-4	not tested	not tested	+	+ +	+ +	+ +
NP-5	no gel	trace	+	+	+ +	+ +
HNP-1	no gel	+	+	+ +	+ +	+ +
HNP-2	trace	trace	trace	+	+	+ +
HNP-3	no gel	+	+	+ +	+ +	+ +
HNP-4	no gel	trace	trace	+ +	+	+ +

As seen from the results, all of the protegrins, both synthetic and native, and both in the amidated and
15 nonamidated forms are able to bind sufficiently to LPS to prevent any substantial gel formation at concentrations as low as 2.5 µg/0.2 ml. nPG-1 and nPG-2 are effective at somewhat lower concentrations. The protegrins were substantially more effective than the NP or HNP test
20 compounds; the most effective among these controls was

NP-3a, a peptide whose primary sequence most closely
resembles that of the protegrins.

     In a follow-up experiment, the concentration of LPS was
varied from 0.05-0.25 endotoxin units (E.U.) and synthetic
5   PG-3 amide was used as the test compound. The results are
shown in Table 2.

Table 2			
Endotoxin Units	0.25 E.U.	0.10 E.U.	0.05 E.U.
sPG-3 amide (2.5 µg)	no gel	no gel	no gel
sPG-3 amide (1.0 µg)	no gel	no gel	no gel
sPG-3 amide (0.5 µg)	+ +	+ +	no gel
no added protein	+ +	+ +	+ +

    These results show that since inhibition of gelation
10   can be overcome by increasing the concentration of LPS,
interaction with LPS is responsible for the lack of
gelation, rather than interfering with the gelation enzyme
cascade.

15   Example 5
Activity of Linearalized Forms
    nPG-1 and nPG-3 were converted to linear form using a
reducing agent to convert the disulfide linkages to
sulfhydryl groups, which were then stabilized by alkylating
20   with iodoacetamide.

    The ability of both cyclic and linearalized PG-1 and
PG-3 to inhibit gelation in the standard LAL assay was
assessed then as described in Example 4 and the results are
shown in Table 3.

25

Table 3					
Peptide	5 µg	2.5 µg	1.0 µg	0.25 µg	
nPG-1	no gel	no gel	+ +	+ +	+ +
cam-nPG-1	no gel	no gel	+ +	+ +	+ +
nPG-3	no gel	no gel	+ +	+ +	+ +
cam-nPG-3	no gel	no gel	+ +	+ +	+ +

These results show that the linearalized and cyclic
forms of the protegrins are equally capable of inhibiting
gelation and binding to endotoxin.

The antimicrobial activity of the linearalized forms
5   was also compared with that of the native protegrins. Both
linearalized and cyclic forms of the protegrins tested
continue to show antimicrobial activity, although the
effectiveness of these peptides as antimicrobials depends on
the nature of the target organism and on the test
10   conditions. The antimicrobial activity of native PG-1 and
its linearalized form (cam-PG-1) and PG-3 and its
linearalized form (cam-PG-3) were tested according to the
procedure set forth in Example 1 as described by Lehrer,
R.I. et al. J Immunol Meth (1991) 137:167-173. The results
15   are set forth in Figures 6a-6f.

Figures 6a and 6b show the antimicrobial activity of
these peptides in the concentration range 20 μg/ml-125 μg/ml
with respect to E. coli ML-35P either in 10 mM phosphate-
citrate buffer, pH 6.5 (Figure 6a) or in the presence of
20   this buffer plus 100 mM NaCl (Figure 6b). Both protegrins
showed strong antimicrobial activity with respect to this
organism; the linear form was slightly more potent in the
presence of buffer alone than was the cyclic form; on the
other hand, the cyclic form was more potent than the linear
25   form under isotonic conditions.

Figures 6c and 6d show the antimicrobial effect with
respect to L. monocytogenes. In Figure 6c where the above-
mentioned buffer alone was used, both cyclic and
linearalized forms of the protegrins showed strong
30   antimicrobial activity and both were approximately equally
effective over the concentration range tested (20 μg/ml-125
μg/ml).

Figure 6d shows the effect with respect to
L. monocytogenes in the presence of this buffer plus 100 mM
35   NaCl over the same concentration range. The cyclic form
retained strong antimicrobial activity with a slightly
greater concentration dependence. Linearalization appeared

to lower the activity appreciably although high
concentrations were still able to show an antimicrobial
effect.

The yeast *C. albicans* was tested with the results shown
5   in Figures 6e and 6f. Figure 6e shows that all forms of
these protegrins were antimicrobial in a dose-dependent
manner over the above concentration range when tested in the
presence of 10 mM phosphate buffer alone, although the
linearalized peptides were very slightly less effective.
10   Figure 6f shows the results of the same assay run in the
presence of buffer plus 100 mM NaCl. While the cyclized
forms retained approximately the same level of antimicrobial
effect, the activity of the linearalized forms was greatly
diminished so that at concentrations below 100 µg/ml of the
15   protegrin, virtually no antimicrobial effect was seen.
However, at higher concentrations of 130 µg/ml, a moderate
antimicrobial effect was observed.

Thus, depending on the target microorganism and the
conditions used, both the cyclized and linearalized forms of
20   the protegrins have antimicrobial activity.

Example 6
Antimicrobial Activity Under Conditions
Suitable for Treatment of the Eye

25   Contact lens solutions are typically formulated with
borate buffered physiological saline and may or may not
contain EDTA in addition. Protegrins in the form of the
synthetic PG-3 amide and synthetic PG acid were tested
generally in the assay described in Example 1 wherein all
30   underlay gels contain 25 mM borate buffer, pH 7.4, 1% (v/v)
tryptocase soy broth (0.3 µg/ml TSB powder) and 1% agarose.
Additions included either 100 mM NaCl, 1 mM EDTA or a
combination thereof. Other test compounds used as controls
were the defensin NP-1 and lysozyme. Dose response curves
35   were determined.

Table 4 shows the estimated minimal bacteriocidal
concentrations in µg/ml of the various test compounds.

Table 4				
ESTIMATED MINIMAL FUNGICIDAL CONCENTRATIONS (µg/ml)				
Peptide	buffer	+ EDTA	+ NaCl	+ EDTA & NaCl
sPG-3 amide	13.0	9.5	4.1	3.1
sPG-3 acid	15.0	9.5	4.6	3.7
NP-1	35.0	45.0	>200	>200
lysozyme	75.0	45.0	>200	>200

Although protegrins are somewhat less active in 25 mM
borate buffered saline than in 25 mM phosphate buffer, the
5   antimicrobial activity is enhanced by adding physiological
saline and modestly enhanced by 1 mM EDTA, as shown in the
table.

A similar test was run with *Candida albicans* as the
target organism with the results shown in Table 5, which
10   also shows estimates of minimal fungicidal concentrations.

Table 5			
ESTIMATED MINIMAL FUNGICIDAL CONCENTRATIONS (µg/ml)			
Peptide	25 mM borate buffer	borate buffer +120 mM NaCl	borate buffer +EDTA & NaCl
nPG-3	32.0	9.0	8.0
sPG-3 amide	19.0	7.7	7.0
sPG-3 acid	19.0	9.2	9.3
NP-1	23.0	60.0	65.0
HNP-1	25.0	>200	>200

Table 6 shows results of similar experiments conducted
with *L. monocytogenes* as the target.
15

Table 6			
ESTIMATED MINIMAL BACTERICIDAL CONCENTRATIONS (µg/ml)			
Peptide	25 mM borate buffer	borate buffer +120 mM NaCl	borate buffer +EDTA & NaCl
nPG-3	25.0	7.0	5.7
sPG-3 amide	21.0	5.7	5.2
sPG-3 acid	30.0	7.0	7.0
NP-1	20.0	11.0	3.8
HNP-1	11.0	>200	>200

The results shown indicate that these compounds are
capable of exerting their antimicrobial effects under
conditions typically associated with conditions suitable for
5   eye care products.

                        Example 7
             Recovery of cDNA Clones and of a
              New Protegrin-Encoding cDNA
10   cDNA Generation and PCR Amplification.
        Total RNA was extracted from the bone marrow cells of a
young red Duroc pig with guanidinium thiocyanate. One μg of
total RNA was used to synthesize the first strand cDNA, with
20 pmol Oligo(dT) primer and 200 U Moloney-murine leukemia
15   virus (M-MLV) reverse transcriptase (Clontech Laboratory,
Palo Alto, CA) in a total reaction volume of 20 μl. Two PCR
primers were prepared. The sense primer
(5'-GTCGGAATTCATGGAGACCCAGAG (A or G) GCCAG-3') corresponded
to the 5' regions of PG-2 and PR-39 cDNA and contained an
20   EcoRI restriction site. The antisense primer (5'-GTCGTCTAGA
(C or G) GTTTCACAAGAATTTATTT-3') was complementary to 3'
ends of PG-2 and PR-39 cDNA immediately preceding their
poly A tails and contained an XbaI restriction site. PCR
was carried out in a 50 μl volume using 1/10 volume of the
25   above pig cDNA as template, 25 pmol primers and 2.5 units of
AmpliTaq DNA polymerase (Perkin Elmer-Cetus). The reaction
was run for 30 cycles, with 1 min denaturation (94°C) and
annealing (60°C) steps and a 2 min extension step (72°C) per
cycle.
30   cDNA Cloning and Sequencing. The amplified cDNA was
fractionated by preparative agarose electrophoresis and
stained with ethidium bromide. The main fragment was cut
out, digested with EcoR I and Xba I endonucleases (New
England Biolabs, Beverly, MA), subcloned into a M13mp18
35   bacteriophage vector, and transformed into E. coli XL1-Blue
MRF' competent cells (Stratagene, La Jolla, CA). DNA
sequencing was performed with a kit (U.S. Biochemical Corp.,

Cleveland, OH). Nucleotide and protein sequences were
analyzed with PC-GENE (Intelligenetics, Palo Alto, CA).

Northern blots. Ten µg of total RNA was denatured in
50% formamide, separated by electrophoresis through 1%
5   agarose gels in 0.62 M formaldehyde, and blotted onto
GeneScreen Plus membranes (DuPont, Boston, MA) by capillary
transfer. The membrane was baked at 80°C for 2 h, and
hybridized with ^{32}P-labeled probe in rapid hybridization
buffer (Amersham, Arlington Height, IL).

10   The results of sequencing the various clones encoding
the various protegrins is summarized in Figure 7. The cDNA
sequences of protegrins PG-1, PG-3 and PG-4 contain 691
bases as had previously been shown for PG-2 by Storici, P.
et al. Biochem Biophys Res Comm (1993) 196:1363-1368. The
15   cDNAs show an upstream sequence encoding 110 amino acids
which appears identical for all protegrins. Additional
differences, which are quite slight in nature, are shown in
Figure 7.

The analysis showed the presence of the protegrin PG-4
20   having an amino acid sequence of Formula (1) wherein A_{10} is a
small amino acid and A_{11} is a hydrophobic amino acid as
distinguished from the previously known protegrins where
these residues are basic. The amino acid sequence of PG-4
is therefore RGGRLCYCRGWICFCVGRG, wherein 1, 2, or 3 amino
25   acids at the N-terminus may be deleted.

Additional clones were obtained by amplifying reverse
transcribed porcine bone cell RNA using an upstream primer
that corresponds to the 5' end of PG-2 and another cathelin-
associated peptide, PR39, (Agerbeth B et al., Eur J Biochem
30   (1991) 202:849-854; Storici, P et al., Biochem Biophys Res
Com (1993) 186:1058-1065) and downstream primer that matches
the region immediately preceding the poly A region. The
resulting approximately 0.7 kb PCR product was subcloned
into M13mp18 and recombinant plaques were chosen for
35   purification and sequencing. In this manner, the sequences
for the precursors of PG-1, PG-3 and PG-4 were recovered.
All of these peptides are encoded by a nucleotide sequence

which encodes a precursor containing additional amino acid sequence upstream of A_1 of the compound of formula 1 (as shown for PG-4 in Figure 7).

<div align="center">

Example 8

</div>

Recovery of Genomic DNA Encoding PG-1, PG-3, and PG-5

High molecular genomic DNA was purified from pig white blood cells with the QIAGEN blood DNA kit (QIAGEN, Chatsworth, CA). To amplify protegrin (PG) genes, PCR as performed using genomic DNA as a template.

The sense primer (5'-GTCGGAATTCATGGAGACCCAGAG(A or G)GCCAG-3') corresponded to the 5' regions of PG cDNAs, of Example 7 and provided an EcoRI restriction site. The antisense primer (5'-GTCGTCTAGA(C or G)GTTTCACAAGAATTTATTT-3') was complementary to 3' ends of PG cDNAs immediately preceding their poly(A) tails and provided an XbaI restriction site. The reaction was carried out in a total volume of 50 μl, which contained 200 ng of purified pig genomic DNA, 25 pmoles of each primer, 1 μl of 10 mM dNTP, 5 μl of 10X PCR buffer (200 mM Tris-HCl, 100 mM(NH$_4$)$_2$, 20 mM MgSO$_4$, 1% Triton X-100, 0.1% BSA), and 2.5 units of cloned Pfu DNA polymerase (Stratagene, La Jolla, CA). Thirty cycles were performed, each with 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, 2 min of primer extension at 72°C, and a final extension step at 72°C for 10 min.

The amplified PCR product was digested with EcoRI and XbaI, excised from the agarose gel, purified, and ligated into pBluescript KS+ vector (Stratagene, La Jolla, CA) that had been digested with EcoRI and XbaI and purified. Both strands of DNA were sequenced by the dideoxy method using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH), pBluescript universal primers and specific oligomer primers based on PG genomic and cDNA sequences. Computer analysis of the DNA sequences was performed using the PC-Gene Program (Intelligenetics, Palo Alto, CA).

A PCR product of about 1.85 kb was confirmed as protegrin-related by hybridization with a protegrin-specific oligonucleotide probe complementary to nucleotides 403-429 of the protegrin cDNA sequences. The PCR product was then

5   subcloned into pBluescript vector, and recombinant plasmids were subjected to DNA purification and sequencing. Gene sequences for three different protegrins were identified PG-1, PG-3 and PG-5. The nucleotide sequences and deduced amino acid sequences are shown in Figure 8.

10   Comparison of protegrin cDNAs and genes revealed that the coding regions of protegrin genes consisted of four exons, interrupted by three introns (Figures 8 and 9). The first exon contained the 5' noncoding region and codons for the first 66 amino acids of the protegrin prepropeptide,

15   including a 29 residue signal peptide and the first 37 cathelin residues. Exons II and III were relatively small, only 108 and 72 bp respectively, and together contained the next 60 cathelin residues. The final two cathelin residues were on Exon IV, and were followed by the protegrin

20   sequences. The exon-intron splice site sequences are shown in Table 7, and conform to the consensus rule: all introns end on an AG doublet, preceded by a T/C rich stretch of 8-12 bases, while all introns start with GT, followed predominantly by A/G A/G G sequence.

25

Table 7					
Exon-Intron Structure of the PG-1 Gene					
Exon	Size	5' splice donor	Intron	Size	3' splice acceptor
1	? + 198	AAGGCCgtgagtcg	1	405	ttgaccagGACGAG
2	108	AACGGGgtgaggct	2	152	ccttccagCGGGTG
3	72	AATGAGgtgagtgg	3	596	ggtcacagGTTCAA
4	313				

The highly conserved cathelin region spans exons I-IV and Exon IV contains the full sequence of the mature protegrin peptide followed by an amidation consensus

30   sequence, a 3' untranslated region, and the putative polyadenylation site. The three introns range in size from

152 to 596 bp. If the protegrin genes are representative of
other cathelin-like genes, the third intron of cathelin-
associated peptides will be found to separate all but the
last two residues of the highly conserved cathelin region
5   from the variable antimicrobial peptides encoded in Exon IV.
Such a layout would favor recombination mechanisms involving
association of diverse Exon IVs with the first three exons
specifying cathelin containing prepro-regions.

The family of naturally occurring protegrins thus
10   contains at least 5 members. Figure 10 shows a comparison
of the amino acid sequences of the five protegrins found so
far in porcine leukocytes. There is complete homology in
positions 1-3, 5-9, 13 and 15-16.

Homology search of protegrin genes against the
15   EMBL/GenBank identified no significantly homologous genes.
More specifically, the gene structures and nucleotide
sequences of protegrins were very different from those of
defensins, which contain three exons in myeloid defensin
genes, and two exons in enteric defensin genes. As
20   expected, the search yielded the large family of cDNAs
corresponding to cathelin-associated bovine, porcine and
rabbit leukocyte peptides.

To assess protegrin-related genes further, we screened
a porcine genomic library of approximately 2.3 x 10^5 clones
25   in EMBL-3 SP6/T7 with the ^{32}P-labeled protegrin cDNA, and
identified 45 hybridizing clones.

A porcine liver genomic library in EMBL3 SP6/T7 phages
was purchased from Clontech (Palo Alto, CA). *E. coli* strain
K803 was used as a host, and DNA from phage plaques was
30   transferred onto nylon membranes (DuPont, Boston, MA). The
filters were hybridized with ^{32}P-labeled porcine 691 PG-3
cDNA. The filters were washed several times, finally at 60°C
in 0.1x SSC and 0.1% SDS, and exposed to x-ray film with an
intensifying screen at -70°C. Positive clones were subjected
35   to two additional rounds of plaque purification at low
density.

DNA purified from hybridizing clones was digested with various restriction endonucleases (New England Biolabs, Beverly, MA), fractionated on 0.8% agarose gels, and transferred onto GeneScreen Plus membrane (DuPont, Boston,

5   MA). The hybridization probes were labeled with ^{32}P and included porcine PG-3 cDNA, and 5'-labeled protegrin-specific oligonucleotide complementary to nt 403-429 of PG-1, 2 and 3 cDNAs. For the cDNA probe, the hybridization and washing conditions were carried out as for the library

10 screening. For the oligonucleotide probe, the membranes were washed at 42°C in 0.1x SSC, 0.1% SDS.

Southern blot analysis was carried out with purified DNA from positive clones by hybridization with protegrin cDNA and a protegrin specific oligonucleotide complementary

15 to nt 403-429 of protegrin cDNA sequences. Although all of the clones hybridized with the complete cDNA probe, only about half of them hybridized with the protegrin-specific probe. A specific oligonucleotide probe for porcine prophenin, another cathelin-associated porcine leukocyte-

20 derived antimicrobial peptide, hybridized to several of the nonprotegrin clones. These results confirm a) that the conserved proregion homologous to cathelin is present within the same gene as the mature antimicrobial peptides and is not added on by posttranscriptional events, and b) that the

25 protegrins account for about half of the cathelin-related genes in the pig.

A synthetic peptide corresponding to the amino acid sequence of PG-5 was prepared and tested with respect to antimicrobial activity against *E. coli*, *L. monocytogenes* and

30 *C. albicans*. The results were compared to those obtained with a synthetically prepared PG-1. The results are shown in Figures 11a-11c. As shown in these graphical representations of the results, PG-5 has comparable antimicrobial activity to PG-1 against all three organisms

35 tested.

Example 9

<u>Preparation of *EnantioPG-1*</u>

Using standard solid phase techniques, a protegrin
having the amino acid sequence of PG-1, but wherein every
amino acid is in the D form was prepared. This form of
5   protegrin was tested against *E. coli*, *L. monocytogenes*, *C.
albicans* and other microbes in the absence and presence of
protease and otherwise as described for the radiodiffusion
assay in agarose gels set forth in Example 1. The results
are shown in Figures 12a-12g.

10   Figure 12a shows that both native PG-1 and *enantioPG-1*
in the absence of protease are equally effective in
inhibiting the growth of *E. coli*. Figure 12b shows that
neither trypsin nor chymotrypsin inhibits the antibacterial
effect of *enantioPG-1*. Figure 12c shows that in the
15   presence of these proteolytic enzymes, the ability of native
PG-1 to inhibit the growth of *L. monocytogenes* is adversely
affected, although, as shown in Figure 12d, in the absence
of these proteases PG-1 is comparably active to an
enantioPG-1.

20

<u>Example 10</u>

<u>Activity of the Protegrins Against STD Pathogens</u>

Table 8 summarizes the activity of the protegrin PG-1
as compared to the defensin HNP-1 against growth of STD
25   pathogens. In these results, "active" means that the
peptide was effective at less than 10 μg/ml; moderately
active indicates that it was active at 10-25 μg/ml; and
slightly active means activity at 25-50 μg/ml. If no effect
was obtained at 50-200 μg/ml the compound was considered
30   inactive.

Table 8		
Activity against human STD pathogens	Pr tegrin PG-1	Defensin HNP-1
HIV-1	Active	Slightly active
Chlamydia trachomatis	Active	Slightly active
Treponema pallidum	Active	Inactive
Neisseria gonorrhoeae	Active	Inactive
Trichomonas vaginalis	Moderately active	Inactive
Herpes simplex type 2	Moderately active	Slightly active
Herpes simplex type 1	Inactive	Slightly active
Hemophilus ducreyi	Not tested	Not tested
Human papilloma virus	Not tested	Not tested

Chlamydia trachomatis

Unlike other bacteria associated with STDs, Chlamydia
requires an intracellular habitat for metabolic activity and
5   binary fission. The life cycle is as follows: there is an
extracellular form which is a metabolically inactive
particle somewhat sporelike in its behavior, referred to as
an elementary body (EB). The EB attaches to the host cell
and is ingested to form an internal vacuolar space often
10   called an "inclusion". The bacterium reorganizes to the
delicate reticulate body (RB) which is noninfective but
metabolically active and which over a 48-72 hour period
undergoes reformation to the EB state. The EBs are then
released from the cell. Rather than a peptidoglycan layer,
15   Chlamydia contains multiple disulfide linkages in cysteine-
rich proteins for protection in the EB stage.

The protegrins of the invention were tested for their
antimicrobial activity against Chlamydia using the "gold
standard" chlamydial culture system for clinical specimens
20   described by Clarke, L.M. in Clinical Microbiology
Procedures Handbook II (1992), Isenberg, H.T. Ed. Am. Soc.
Microbiol. Washington, D.C.; pp. 8.0.1 to 8.24.3.9.
Briefly, McCoy cells (a mouse cell line) in cycloheximide

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EMEM with 10% fetal bovine serum (FBS) are used as hosts.
Prior to chlamydial inoculation, the maintenance medium is
aspirated without disruption of the cell layer and the cell
layer is maintained on a cover slip in a standard vial.

5   Each vial is then inoculated with 100-300 μL inoculum and
centrifuged at 3500 x g for one hour at 20°C. The fluid is
then aspirated and 1 ml of EMEM is added. The vials are
capped and incubated at 37°C for 48 hours. After 48 hours
the medium is again aspirated, coverslips are rinsed twice

10   with PBS and fixed with 300 μL EtOH for 10 minutes. The
EtOH is aspirated and the vials are allowed to dry; then one
drop PBS plus 30 μL *Syva* Microtrak monoclonal antibody to
the major outer membrane protein of *Chlamydia* is added for
staining. After 37°C incubation for 30 minutes, the cells

15   are washed with distilled water and examined for inclusions
which are easily recognizable as bright, apple-green-
staining cytoplasmic vacuoles. They represent the
equivalent of a colony of free-living bacteria on standard
bacterial culture media.

20   In the assays conducted below, *C. trachomatis* serovar
L2 (L2/434Bu) described by Kuo, C.C. *et al.* in <u>Nongynococcal</u>
<u>Urethritis and Related Infections</u> (1977), Taylor-Robinson,
D. *et al.* Ed. Am. Soc. Microbiol. Washington, D.C., pp. 322-
326 was used. The seed is prepared from a sonicated culture

25   in L929 mouse fibroblast cells, and partially purified by
centrifugation. Since host protein is still present in the
seed aliquots, each seed batch is titered at the time of
preparation with serial ten-fold dilutions to 2×10^{-9}. The
seed containing 9.2×10^{6} IFU/ml is thawed quickly at 37°C

30   and diluted to 10^{-2} with sucrose/phosphate salts/glycine to
produce IFU of about 200 after room temperature
preincubation and to dilute background eukaryotic protein.

     In the initial assays, the peptides to be tested were
prepared as stock solutions in 0.01% glacial acetic acid.

35   100 μL of the diluted chlamydial seed was aliquoted into 1.5
ml eppendorf tubes and 200 μL of the antibiotic peptide was

added per tube. Aliquots of the peptide stock (and
controls) were incubated with the seed at room temperature
for one hour, two hours and four hours. About 10 minutes
before the end of each incubation period, maintenance media
5   were aspirated from the McCoy vials in preparation for
standard inoculation and culture. Culture was then
performed in the presence and absence of the peptides; in
some cases, the peptides were added to final concentration
in the culture media in addition to the preculture
10   incubation. The test was evaluated microscopically.

The results using 50 µg of protegrin per addition were
dramatic. In control cultures, where no peptides were
added, 222-460 inclusions were counted. In all protocols
where protegrin was added either before the *Chlamydia* seed
15   was added to the cells or both before and after, no
inclusions were found. Similar results were obtained with
20 µg additions of tachyplesin. The defensins NP-1 and
HNP-1 had lesser protective effects. In summary, the
protegrins tested show antimicrobial against *Chlamydia*.
20   In the next series of experiments, various
concentrations of protegrin (1 µg, 12.5 µg, 25 µg and 50 µg)
were used in the two-hour preincubation. Concentrations as
low as 12.5 µg lowered the number of inclusions to zero.
Even at a concentration of 1 µg/ml, the number of inclusions
25   was lowered dramatically from about 110 to about 30.

In the next set of experiments, the effect of the
presence of serum was tested. The *Chlamydia* seed was
preincubated for two hours with and without 10% FBS and also
with or without protegrin at 25 µg. Protegrin was highly
30   effective both with and without serum, whereas human
defensin HNP-2, used as a control, was reasonably effective
in the absence of serum but only marginally effective in its
presence.

The experiments were repeated but adding 25 µg of
35   protegrin one after the start of the chlamydial culture,
i.e., after centrifugation and final medium mix and one hour
into the beginning of the 48-hour culture period. Protegrin

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reduced the number of inclusions by approximately 57% from untreated controls although HNP-2 was completely ineffective. Finally, the protegrin (at 25 µg) was added to the chlamydial seed and the mix then immediately cultured.

5   In this case, without preincubation and without the one-hour post-infection gap, protegrin was minimally effective without or without serum.

The effect of serum is particularly important since for a topical agent to be effective in combatting *Chlamydia*

10   infection, it must act in the presence of serum.

In addition, there are several mouse-based models for *Chlamydia* infection which can be used to assess the efficacy of the protegrins. These include those described by Patton, D.L. *et al.* in <u>Chlamydial Infections</u> (1990) Bowie, W.R. *et*

15   *al.* Eds. Cambridge University Press NY pp. 223-231; Swenson, C.E. *et al.* <u>J. Infect. Dis.</u> (1983) pp. 1101-1107, and Barron, A.L. *et al.* <u>J. Infect. Dis.</u> (1981) <u>143</u>:63-66.

Neisseria gonorrhoeae

20   In more detail, the ability of the protegrins to inhibit *N. gonorrhoeae* was tested by a modification of the method of Miyasaki et al., <u>Antimicrob Agent Chemother</u> (1993) <u>37</u>:2710-2715. Nonpiliated transparent variants of strains FA 19 and F 62 were propagated on GCB agar plates containing

25   glucose and iron supplements overnight at 37°C under 3.8% V/V CO_2. These strains were chosen for their adaptability to the assay.

The overnight growth is removed from the agar plate and suspended in GCB broth containing supplements and sodium

30   bicarbonate and grown with shaking at 37°C to mid log phase. The culture is diluted 1:100 in GCB broth to give about 10^6 CFU/ml and serial dilutions were plated onto GCB agar.

The peptides are dissolved in 0.01% v/v acetic acid to give a 1 mg/ml stock solution and serially diluted. Ten µl

35   of each dilution is added to a sterile polystyrene tube containing 90 µl of diluted bacteria and the tubes are shaken at 37°C for 45 minutes. The contents are serially

diluted 1:10 and plated on to GCB agar plates which are incubated in a CO_2 incubator. CFU are counted after 24 hours and the log bactericidal activity calculated.

5   Native PG-1, synthetic PG-1, synthetic PG-3 amide and synthetic PG-3 without amidation all gave over a 5 log reduction in CFU per ml in this assay. Native PG-2 (containing 16 amino acids) gave a 2.6 fold reduction.

In addition *enantio*PG-1, the unidisulfide PG-1 (C_6-C_{15}), and unisulfide PG-1 (C_8-C_{13}) gave over a 5-fold log reduction 10   in CFU/ml in this assay.

Treponema pallidum

Bacteriocidal activity against this organism, which is the etiologic agent of syphilis, was also tested. Peptides 15   were evaluated at a series of concentrations of 1.758 μg to 56.25 μg in 90 μl of unheated normal rabbit serum. The serum served as a nutrient for the spirochetes to allow their survival during incubation as well as providing a source of complement. Ten μl of a suspension of *T. pallidum* 20   containing about 5 x 10^7/μl organisms was added to each tube and the mixtures with the appropriate peptides were incubated at 34°C under 95% N_2 and 5% CO_2. At time zero, just prior to incubation, 4 hours and 16 hours, 25 randomly selected organisms were examined for the presence or absence 25   of motility. The 50% immobilizing end point (IE_{50}) was calculated to indicate the concentration needed to immobilize 50% of the spirochetes. In the presence of PG-1, the IE_{50} at 0 and 4 hours was 2.717 μg and < 1.758 μg, respectively. Tachyplesin IE_{50}'s were 5.231 μg and 2.539 μg 30   for 0 and 4 hours. This was in contrast to HNP and NP preparations which showed little immobilizing ability.

Herpes Simplex Virus

Using viral stocks prepared in VERO cells, grown in 35   minimal essential medium (MEM) with 2% fetal calf serum, the effect of various peptides on HSV 1 MacIntyre strain, a pool of ten clinical HSV 1 isolates, HSV-2G, and a pool of ten

clinical HSV 2 isolates, all sensitive to 3 μM acyclovir
were tested. Two fibroblast cell lines, human W138 and
equine CCL57, were used as targets and tests were done by
direct viral neutralization and delayed peptide addition.

5   In the direct neutralization format, the virus was
preincubated with the peptides for 90 min before it was
added to the tissue culture monolayers. In the delayed
peptide addition format, the virus was added and allowed 50
min to adsorb to the target cells, then the monolayers were
10   washed and peptides were added for 90 min. Finally, the
monolayer was washed to remove the peptide and the cells
were fed with peptide-free MEM and cultured until the
untreated infected monolayers exhibited 4+ cytopathic effect
(CPE) (about 60 hours).

15   Antiviral activity was seen in both formats, but was
more pronounced with the delayed peptide addition mode. In
experiments performed with W138 and CCL57 cells in the
direct neutralization format, PG-1 completely prevented HSV-
2G from causing CPE at concentrations of 50 μg/ml and 25
20   μg/ml, but these concentrations afforded no protection
against HSV-1, which produced 4+ CPE.

          In the delayed peptide addition format, PG-1 completely
prevented CPE by HSV-2G at 35 μg/ml and 50 μg/ml and it also
fully protected against the clinical HSV-2 pool at both
25   concentrations.

          Thus, PG-1 protected human and animal cells from
infection by laboratory and clinical strains of HSV-2, even
when the peptides were added as late as 60 min after the
virus had been introduced into the cell culture.

30

Trichomonas vaginallis

          Trichomonas vaginallis strain C1 (ATCC 30001) was grown
as described by Gorrell, T.E. et al, Carlsberg Res Comm
(1984) 49:259-268. In experiments performed in RPMI + 1%
35   heat-activated fetal calf serum, within a few minutes after
exposure to 50 μg/ml PG-1, *T. vaginallis* (heretofore
vigorously motile) became stationary. Soon thereafter, the

organisms became permeable to trypan blue, and, over the
ensuing 15-30 minutes, lysed. As expected, such organisms
failed to grow when introduced into their customary growth
medium (Diamond's medium). Organisms exposed to 25 µg/ml of
5   PG-3 retained their motility.

　　　　Initial studies with two highly metronidazole-resistant
clinical isolates of *T. vaginallis*, strains MR and TV showed
both were susceptible to PG-1, including the C_8-C_{13} and C_6-
C_{15} uni-disulfides and *enantio*PG-1 at concentrations of 100
10   and 50 µg/ml.

Example 11
Antiretroviral Activity

　　　　Both synthetic and native PG-1 and native PG-2 were
15   tested for antiviral activity against strains of HIV using
the method described in Miles, S.A. et al., Blood (1991)
78:3200-3208. Briefly, the mononuclear cell fraction is
recovered from normal donor leukopacs from the American Red
Cross using a Ficoll-hypaque density gradient. The
20   mononuclear cells are resuspended at 1 x 10^6 cells per ml in
RPMI 1640 medium with 20% fetal bovine serum, 1% penn/strep
with fungizone and 0.5% PHA and incubated 24 hours at 37°C
in 5% CO_2. The cells are centrifuged, washed and then
expanded for 24 hours in growth medium.
25   　　　　Non-laboratory adapted, cloned HIV_{JR-CSF} and HIV_{JR-FL}
were electroporated into the human peripheral blood
mononuclear cells prepared as described above. Titers were
determined and in general, multiplicities of infection (MOI)
of about 4,000 infectious units per cell are used (which
30   corresponds to 25-40 picograms per ml HIV p24 antigen in the
supernatant).

　　　　In the assay, the HIV stocks prepared as above were
diluted to the correct MOI and the PBM are added to 24 well
plates at a concentration of 2 x 10^6 per ml. One µl total
35   volume is added to each well. The peptide to be tested is
added in growth medium to achieve the final desired
concentration. Then the appropriate number of MOI are

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added. To assay viral growth, 200 µl of supernatant is
removed on days 3 and 7 and the concentration of p24 antigen
is determined using a commercial assay (Coulter Immunology,
Hialeah, Florida). Controls include duplicate wells
5   containing cells alone, cells plus peptide at 5 µg/ml cells
with virus but not peptide and cells with virus in the
presence of AZT at 10^{-5} M - 10^{-8} M.

Using this assay, it was demonstrated that both natural
and synthetic PG-1 completely inhibit HIV infection at
10   concentrations between 1-5 µg/ml; IC_{90} was < 5 µg/ml. The
time of addition of peptide was then varied. Cells
pretreated for 2 hours prior to addition of virus, at the
time of addition of virus, or 2 hours after infection showed
antiviral activity for the peptide. However, if PG-1 was
15   added 24 hours after infection, there was no antiviral
activity.

Further, PG-2 shows similar activity but at a level
approximately 5-fold less. Alternative antibiotics such as
human defensins and rabbit defensins lacked potent activity
20   in this assay. The results were similar for both HIV_{JR-CSF}
and HIV_{JR-FL} which are non-laboratory adapted isolates
(Koyanagi, Y.S. et al, <u>Science</u> (1987) <u>236</u>:819-822).

The protegrins show similar activity with respect to
other retroviruses.
25

Example 12
Preparation of Modified Protegrins: Kite and Bullet Forms
The kite and bullet forms of PG-1 wherein all X are
alanine were synthesized using conventional Fmoc chemistry.
30   The crude synthetic peptide was reduced by adding
dithiothreitol (DTT) equal in weight to the synthetic
peptide which had been dissolved at 10 mg peptide/ml in a
solution containing 6 molar guanidine HCl, 0.5 molar tris
buffer, and 2 mM EDTA, pH 8.05 and incubated for two hours
35   at 52°C under nitrogen. The mixture was passed through a
0.45 µ filter, acidified with 1/20 (v/v) glacial acidic acid
and subjected to conventional RP-HPLC purification with a

C-18 column. HPLC-purified, reduced synthetic bullet and
kite PG-1 were partially concentrated by vacuum
centrifugation in a speed vac and allowed to fold for 24
hours at room temperature in ambient air in 0.1 M Tris

5   pH 7.7 at low concentration (0.1 mg peptide/ml) to minimize
formation of interchain cystine disulfides. The mixture was
then concentrated and acidified with HOAC to a final
concentration of 5% and subjected to RP-HPLC purification.
        The purity of the final products bullet and kite PG-1

10 was verified by AU-PAGE, analytical HPLC, and FAB-mass spec.
AU-PAGE showed a single band for the final product in each
case. The observed MH+ mass values were 2093 in both cases.

                        Example 13

15   Antimicrobial Activity of the Kite and Bullet Forms
        The kite and bullet PG-1 compounds prepared in Example
12 were tested for antimicrobial activity using the radial
diffusion assay described in Example 1 as published by
Lehrer, R.I. et al., J Immunol Meth (1991) 137:167-173,

20 except that the underlay agars contained 10 mm sodium
phosphate buffer with a final pH of 7.4. As described in
Example 1, 0.3 mg/ml tripticase soy broth powder and 1%
agarose were used as well in the underlay agar. In some
cases 100 mM NaCl or RPMI plus 2.5% normal human serum (NHS)

25 was added to the agar.
        In a first set of determinations, the bullet and kite
forms of PG-1 were tested for antimicrobial activity against
L. monocytogenes, E. faecium (VR) or S. aureus under these
three sets of conditions. Figure 13 shows the result.

30   As shown, the bullet and kite forms were roughly
equally effective against these three bacteria using
standard assay conditions. When 100 mM NaCl was added to
the agar, however, the kite forms appeared slightly less
active than the bullet forms which appear to have slightly

35 enhanced antimicrobial activity against all three stains
except S. aureus under these conditions. Similarly, when
RPMI plus 2.5% NHS were added, the bullet forms were again
more effective than the kite forms. The activity of the kit

form versus *E. faecium* was significantly less under these
conditions.

      As shown in Figure 14, these forms of PG-1 were also
tested against *E. coli*, *K. pneumoniae* and *P. aeruginosa*.
5 All three microorganisms were inhibited by both kite and
bullet forms under standard conditions. This antimicrobial
activity was maintained also at 100 mM NaCl and RPMI plus
NHS.

10   Example 14
                Synthesis of the Snake Form of PG-1
      The snake form of PG-1 wherein all X are alanine was
performed using standard methods by Synpep Inc., Dublin, CA
and the MH+ value in FAB-mass spec was 2031.3 as expected.
15 The snake form was purified to homogeneity by RP-HPLC.

                              Example 15
               Antimicrobial Activity of Snake PG-1
      Snake PG-1 was tested with respect to the same six
20 organisms and using the same conditions as set forth in
Example 13 with respect to the bullet and kite forms of
PG-1. The results are shown in Figures 15 and 16. In this
case, the native two-cystine form of PG-1 (native) was used
as a control. While the snake form shows somewhat superior
25 activity with respect to *L. monocytogenes*, *E. faecium*, and
S. aureus under standard conditions, it is notably less
effective than the native form in the presence of either
100 mM NaCl or RPMI plus NHS. The same pattern is followed,
as shown in Figure 16 when the test organisms are *E. coli*,
30 *K. pneumoniae*, and *P. aeruginosa*.

                              Example 16

           Minimal Inhibitory Concentrations of Protegrins
      The minimal inhibitory concentrations (MICs) of a
35 variety of protegrins were determined against the following
organisms: methicillin resistant *Staphylococcus aureus*

(MRSA), *Pseudomonas aeruginosa* (Psa), vancomycin resistant

Enterococcus fecium (VREF), *Candida albicans* (Candid) and

Escherichia coli (E. Co), and are shown in Table 9.

	Table 9 Peptides with 17-18 Amino Acids					
	SEQUENCE	MRSA	Psa	VREF	Candid	E. Co
IB-247	RGGRLCYCRRRFCVCVGR-OH	1.5	0.11		1.2	0.6
IB-249	RGGGLCYCRRRFCVCVGR-OH				3.29	0.4
IB-223	RGGGLCYCRRGFCVCFGR	1.93	0.14		1.62	
IB-224	RGGGLCYCRRPFCVCVGR	3.1	0.06		7.69	0.15
IB-324	RGGGLCYCRPRFCVCVGR-OH				17.7	3.51
IB-341	RGGRLCYCRXRFCVCVGR-OH (X=NMeG)	5.33	2	1		
IB-342	RGGRLCYCRXRFCVCVGR (X=NMeG)	4	1.67	0.83		
IB-384	RGGRLCYCXGRFCVCVGR (X=Cit)					
IB-398	RGGRVCYCRGRFCVCVGR	8	1			
IB-399	RGGRVCYCRGRFCVCVGR-OH					
IB-218	RGGGLCYCFPKFCVCVGR	3.48	1.2		15.96	
IB-349	RGGRLCYCRXR-Cha-CVCWGR (X=NMeG)					
IB-350	RGGRWCVCRXR-Cha-CYCVGR (X=NMeG)					.
IB-394	RGGRWCVCRGR-cha-CYCVGR					
IB-416	RGGRLCYCRRRFC-NMeV-CVGR					
IB-400	RGGRVCYCRGRFCVCV	8	2			..
IB-401	RGGRVCYCRGRFCVCV-OH	64	1			
	Uni-Disulfide Protegrins					
IB-214	RGGGLCYARGWIAFCVGR	2.1	0.59		32.6	0.81
IB-216	RGGGLCYARGFIAVCFGR	19	14		65.8	3.27
IB-225	RGGGLCYARPRFAVCVGR					
IB-226	RGGGLCYTRPRFTVCVGR	8.7	0.07			1.53
IB-227	RGGGLCYARKGFAVCVGR	>128	0.01			2.65
IB-288	RGGRLCYARRRFAVCVGR-OH		0.05		1.6	0.4
IB-289	RGGRLCYARRRFAVCVGR		0.05		1.6	0.4

5

Claims

1.   A purified and isolated or recombinantly produced
compound of the formula

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \tag{1}$$

and the N-terminal acylated and/or C-terminal amidated
or esterified forms thereof, which is either in the
optionally -SH stabilized linear or in a cystine-bridged
form
        wherein A_1 is a basic amino acid;
        each of A_2 and A_3 is independently a small amino acid;
        each of A_5, A_7, A_{14} is independently a hydrophobic amino
acid;
        A_4 is a basic or a small amino acid;
        each of A_9, A_{12} and A_{16} is independently a basic, a
hydrophobic, a neutral/polar or a small amino acid;
        each of A_{10} and A_{11} is independently a basic, a
neutral/polar, a hydrophobic or a small amino acid or is
proline;
        A_{17} is not present or, if present, is a basic, a
neutral/polar, a hydrophobic or a small amino acid;
        A_{18} is not present or, if present, is a basic, a
hydrophobic, a neutral/polar or a small amino acid, or a
        modified form of Formula (1) and the N-terminal
acylated and/or C-terminal amidated or esterified forms
thereof wherein at least one of the 4 cysteines is
independently replaced by a hydrophobic amino acid or a
small amino acid;
        with the proviso that the compound of Formula (1) must
have a charge of +3 or greater.

2.   The compound of claim 1 which contains two cystine
bridges.

3.   The compound of claim 1 which contains one cystine
bridge, which is C_6-C_{15} or C_8-C_{13}.

4.   The compound of claim 1 which is in the linear
form.

5.   The compound of any of claims 1-4 wherein the
C-terminal carboxyl is of the formula selected from the
group consisting of COOH or the salts thereof; COOR, $CONH_2$,
CONHR, and $CONR_2$ wherein each R is independently
hydrocarbyl(1-6C);
    and/or wherein the amino group at the N-terminus is of
the formula NH_2 or NHCOR wherein R is hydrocarbyl(1-6C);
    and/or wherein each of A_1 and A_9 is independently
selected from the group consisting of R, K and Har;
    and/or wherein each of A_2 and A_3 is selected
independently from the group consisting of G, A, S and T;
    and/or wherein A_4 is R or G;
    and/or wherein each of A_5, A_{14} and A_{16} is independently
selected from the group consisting of I, V, NLe, L and F;
    and/or wherein each of A_7 and A_{12} is independently
selected from the group consisting of I, V, L, W, Y and F;
    and/or wherein A_{10} is R, G or P;
    and/or wherein A_{11} is R or W.

6.   The compound of claim 1 which is selected from the
group consisting of
    <u>PG-1</u>:   RGGRLCYCRRRFCVCVGR
    <u>PG-2</u>:   RGGRLCYCRRRFCICV
    <u>PG-3</u>:   RGGGLCYCRRRFCVCVGR
    <u>PG-4</u>:   RGGRLCYCRGWICFCVGR
    <u>PG-5</u>:   RGGRLCYCRPRFCVCVGR
    <u>PC-39</u>:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-R
    <u>PC-41</u>:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G
    <u>PC-100</u>:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-Y

PC-101: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-T

PC-102: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-A

PC-103: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-L

PC-104: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-I

5   PC-105: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-F

PC-106: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W

PC-108: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R

               R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R

               R-G-G-R-L-C-W-C-R-R-R-F-C-V-C-V-G-R

10   R-G-G-R-L-C-Y-C-R-R-R-W-C-V-C-V-G-R

               R-G-G-R-L-C-Y-C-R-R-R-F-C-W-C-V-G-R

               R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W-G-R

IB-247: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH

IB-249: R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH

15   IB-223: R-G-G-G-L-C-Y-C-R-R-G-F-C-V-C-F-G-R

IB-224: R-G-G-G-L-C-Y-C-R-R-P-F-C-V-C-V-G-R

IB-324: R-G-G-G-L-C-Y-C-R-P-R-F-C-V-C-V-G-R-OH

IB-341: R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R-OH (X=NMeG)

IB-342: R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R (X=NMeG)

20   IB-384: R-G-G-R-L-C-Y-C-X-G-R-F-C-V-C-V-G-R (X=Cit)

IB-398: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R

IB-399: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R-OH

IB-218: R-G-G-G-L-C-Y-C-F-P-K-F-C-V-C-V-G-R

IB-349: R-G-G-R-L-C-Y-C-R-X-R-Cha-C-V-C-W-G-R (X=NMeG)

25   IB-350: R-G-G-R-W-C-V-C-R-X-R-Cha-C-Y-C-V-G-R (X=NMeG)

IB-394: R-G-G-R-W-C-V-C-R-G-R-Cha-C-Y-C-V-G-R

IB-416: R-G-G-R-L-C-Y-C-R-R-R-F-C-NMeV-C-V-G-R

IB-400: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V

IB-401: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-OH

30   PC-49: R-G-G-R-L-C-W-A-R-R-R-F-A-V-C-V-G-R

PC-50: R-G-G-R-L-C-Y-A-R-R-R-W-A-V-C-V-G-R

PC-52: R-G-G-R-L-A-W-C-R-R-R-F-C-V-A-V-G-R

PC-53: R-G-G-R-L-A-Y-C-R-R-R-F-C-V-A-W-G-R

PC-55: R-G-G-R-L-A-W-A-R-R-R-F-A-V-A-V-G-R

35   PC-56: R-G-G-R-L-A-Y-A-R-R-R-W-A-V-A-V-G-R

PC-57: R-G-G-R-L-A-Y-A-R-R-R-F-A-V-A-W-G-R

IB-214: R-G-G-G-L-C-Y-A-R-G-W-I-A-F-C-V-G-R

IB-216: R-G-G-G-L-C-Y-A-R-G-F-I-A-V-C-F-G-R

IB-225: R-G-G-G-L-C-Y-A-R-P-R-F-A-V-C-V-G-R
IB-226: R-G-G-G-L-C-Y-T-R-P-R-F-T-V-C-V-G-R
IB-227: R-G-G-G-L-C-Y-A-R-K-G-F-A-V-C-V-G-R
IB-288: R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R-OH
IB-289: R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R

and the amidated forms thereof either in linear or cystine-bridged form.

7. The compound of any of claims 1-6 wherein all amino acids are in the D-configuration.

8. A recombinant expression system for production of an antimicrobial peptide having the amino acid sequence of the compound of any of claims 1-6 which expression system comprises a nucleotide sequence encoding said peptide operably linked to control sequences for effecting expression.

9. A recombinant host cell modified to contain the expression system of claim 8.

10. A method to produce an antimicrobial or antiviral peptide or intermediate peptide therefor which method comprises culturing the modified host cells of claim 9 under conditions wherein said peptide is produced; and recovering the peptide from the culture.

11. The method of claim 10 which further comprises effecting cystine linkages of said peptide and/or modifying the N-terminus and/or C-terminus of said peptide.

12. A pharmaceutical composition for antimicrobial or antiviral use which comprises the compound of any of claims 1-7 in admixture with at least one pharmaceutically acceptable excipient.

13. A composition for application to plants or plant environments for conferring resistance to microbial or viral infection in plants which comprises the compound of any of
5 claims 1-7 in admixture with at least one environmentally acceptable diluent.

14. A method to prevent the growth of a virus or microbe which method comprises contacting a composition
10 which supports the growth of said virus or microbe with an amount of the compound of any of claim 1-7 effective to prevent said growth.

15. A method to inactivate the endotoxin of gram-
15 negative bacteria, which method comprises contacting said endotoxin with an amount of the compound of any of claims 1-7 effective to inactivate said endotoxin.

16. Antibodies specifically reactive with the compound
20 of any of claims 1-7.

Figure 1

Figure 2
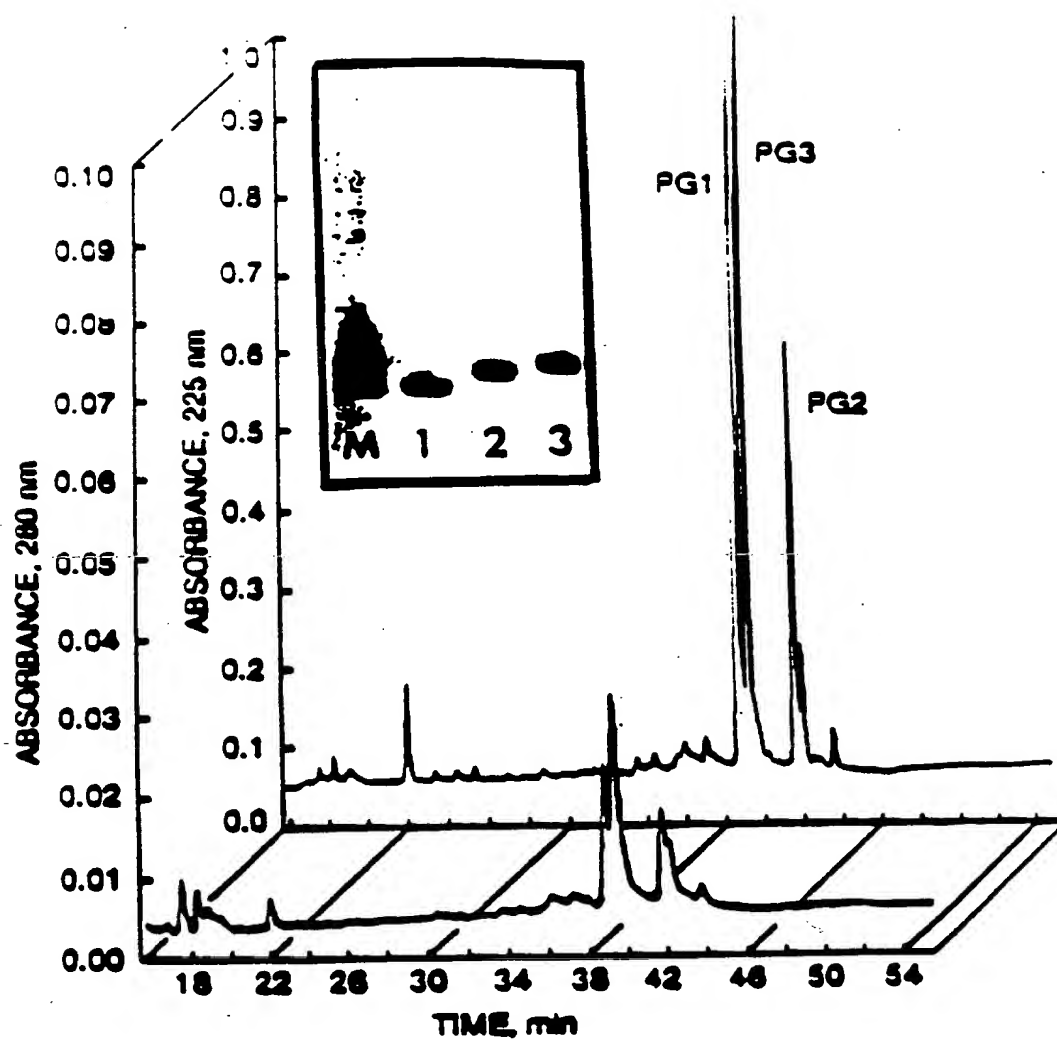
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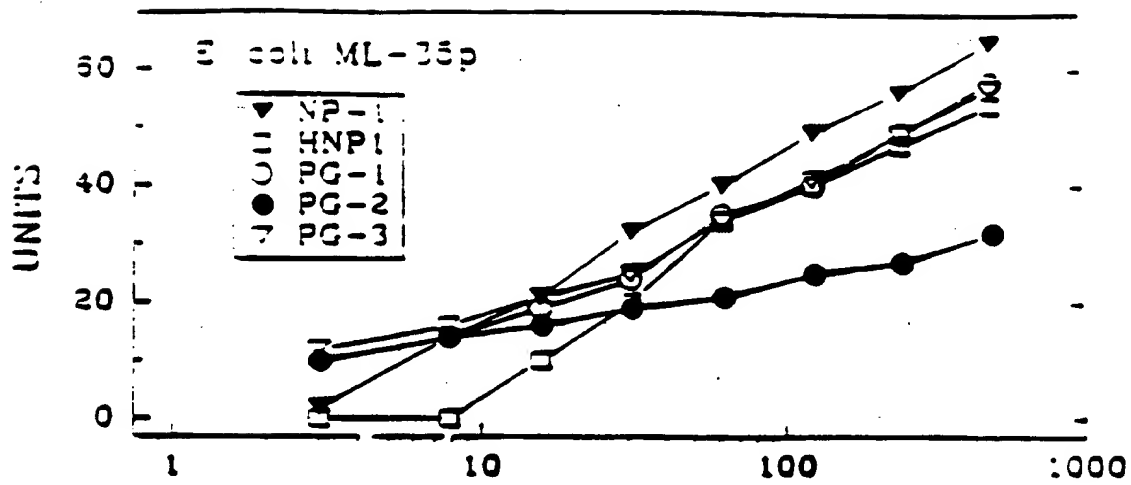
Figure 4a
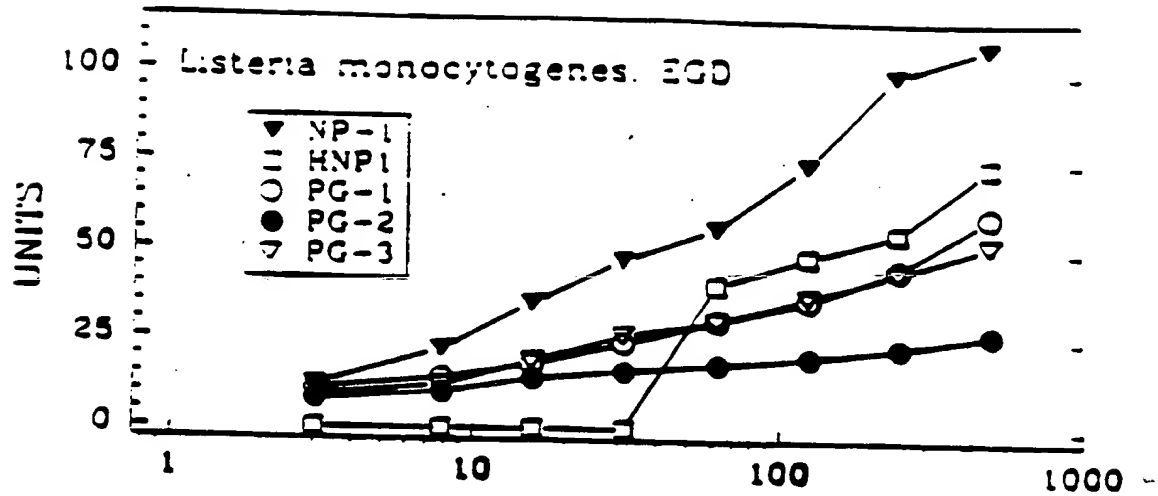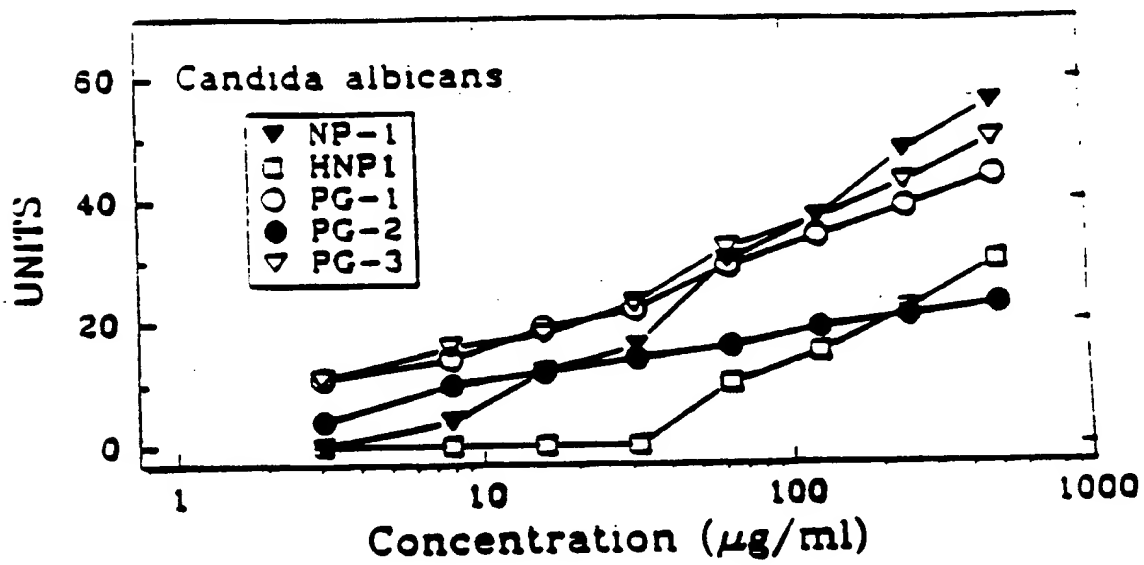
Figure 4b

Figure 4c

We compared the activity of synthetic PG-1 and native human defensin HNP-1 against a mouse-virulent strain of S. aureus. The underlay agar contained 10 mM sodium phosphate buffer and a 1:100 dilution of trypticase soy broth. Neither saline nor serum was present, rendering these conditions especially favorable to defensins. The solid circles represent PG-1, and the open circles represent HNP-1

Staph aureus 930918-3
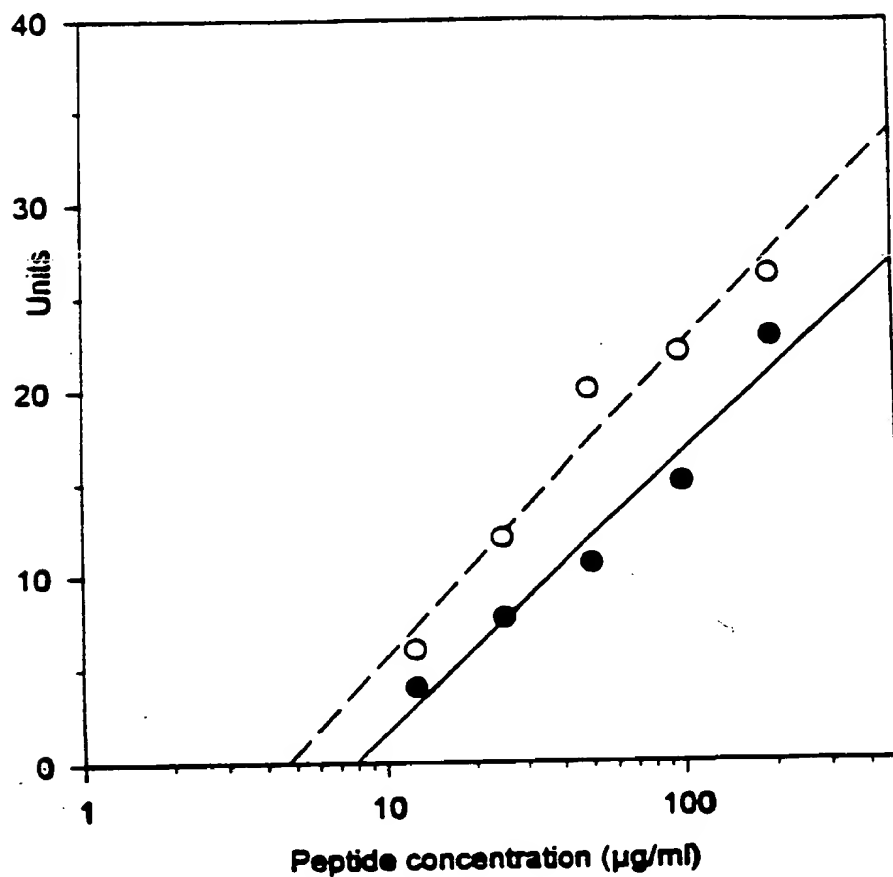
Figure 4d

We compared the activity of synthetic PG-1 and native human defensin HNP-1 against a mouse-virulent strain of *Klebsiella pneumoniae*. The underlay agar contained 10 mM sodium phosphate buffer and a 1:100 dilution of trypticase soy broth. Neither saline nor serum was present, rendering these conditions especially favorable to defensins. The solid circles represent PG-1, and the open symbols represent HNP-1, which was inactive at the conentrations tested here.

K. pneumoniae 2270

Peptide concentration (μg/ml)

Figure 4e

Figure 5a

Figure 5b

Figure 5e

Figure 6a

Figure 6b

Activity of protegrins against L. monocytogenes
in 10 mM phosphate-citrate buffer, pH 6.5.

Figure 6c

Activity of protegrins against L. monocytogenes
in 10 mM phosphate-citrate buffer, pH 6.5
with 100 mM NaCl

Figure 6d

Figure 6e

Figure 6f

ATGGAGACCAGAGAGCCAGCCTGTGCCTGGGCGCTGGTCACTGTGGCTTCTGCTGCTG   60
METGluThrGlnArgAlaSerLeuCysLeuGlyArgTrpSerLeuTrpLeuLeuLeuLeu   20

GCACTCGTGGTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTACAGGGAGGCCGTGCTTCGT   120
AlaLeuValValProSerAlaSerAlaGlnAlaLeuSerTyrArgGluAlaValLeuArg   40

GCTGTGGATCGGCTCAACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCTCCTGGAGCTG   180
AlaValAspArgLeuAsnGluGlnSerSerGluAlaAsnLeuTyrArgLeuLeuGluLeu   60

GACCAGCCGCCCAAGGCCGACGAGGACCCGGGCACCCCGAAACCTGTGAGCTTCACGGTG   240
AspGlnProProLysAlaAspGluAspProGlyThrProLysProValSerPheThrVal   80

AAGGAGACTGTGTGTCCCAGGCCGACCCGGCAGCCCCCGGAGCTGTGTGACTTCAAGGAG   300
LysGluThrValCysProArgProThrArgGlnProProGluLeuCysAspPheLysGlu   100

AACGGGCGGGTGAAACAGTGTGTGGGGACAGTCACCCTGGATCAGATCAAGGACCCGCTC   360
AsnGlyArgValLysGlnCysValGlyThrValThrLeuAspGlnIleLysAspProLeu   120

                                        G³   G⁴
GACATCACCTGCAATGAGGTTCAAGGTGTCAGGGGAGGTCGCCTGTGCTATTGTAGGCGT   420
AspIleThrCysAsnGluValGlnGlyValArgGlyGlyArgLeuCysTyrCysArgArg   140
                                        Gly³   Gly⁴

      A⁴   A²   T²
AGGTTCTGCGTCTGTGTCGGACGAGGATGACGGTTGCGACGGCAGGCTTTCCCTCCCCCA   480
ArgPheCysValCysValGlyArgGly---   149
Trp⁴Ile⁴ Phe⁴   ---²
          Ile²

ATTTTCCCGGGGCCAGGTTTCCGTCCCCCAATTTTTCCGCCTCCACCTTTCCGGCCCGCA   540

                              A² G²
CCATTCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAGGATTTGCAGGCAACTCACCCAG   600

      C⁴
AAGGCCTTTCGGTACATTAAAATCCCAGCAAGGAGACCTAAGCATCTGCTTTGCCCAGGC   660

CCGCATCTGTCAAATAAATTCTTGTGAAACC   691

Figure 7

```
ATGGAGACCCAGAGAGCCAGCCTGTGCCTGGGGCGCTGGTCACTGTGGCTTCTGCTGCTG    60
 M  E  T  Q  R  A  S  L  C  L  G  R  W  S  L  W  L  L  L  L
 G5
GCACTCGTGGTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTACAGGGAGGCCGTGCTTCGT   120
 A  L  V  V  P  S  A  S  A  Q  A  L  S  Y  R  E  A  V  L  R
 G5
GCTGTGGATCGCCTCAACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCTCCTGGAGCTG   180
 A  V  D  R  L  N  E  Q  S  S  E  A  N  L  Y  R  L  L  E  L
GACCAGCCGCCCAAGGCCgtgagtcgggcaggggctcaggagggggctggggggcggggggc   240
 D  Q  P  P  K  A
tgtcccccacccgccccggggctccctgtccctcccctgctcaggctgtccctcctgcc   300

aggaaggcacttgtccctctaaggggggaccccctctgccaggaaaccttcccagagctgg   360

gtgccctgcccgcgtgagagcttcccgccttagcctctgggctgtgggctcagggccctg   420

cacagcctgtgaggcaggagcgggctctgtccctcccctgtgcacccagcaccaagccc...480

agggccaggctcccagcaggggctgcagaggctgctgtctaggtgggggcggggaggggg   540

tgacagatccgaggggaagcctgagcccgagtcccatctccccactttgatccttgacc   600
               A5
agGACGAGGACCCGGGCACCCCGAAACCTGTGAGCTTCACGGTGAAGGAGACTGTGTGTC   660
   D  E  D  P  G  T  P  K  P  V  S  F  T  V  K  E  T  V  C
CCAGGCCGACCCGGCAGCCCCCGGAGCTGTGTGACTTCAAGGAGAACGGGgtgaggctgg   720
 P  R  P  T  R  Q  P  P  E  L  C  D  F  K  E  N  G
gggctggggggcgctggcggatgcttcccaaggagctgaacaggagagcctgctggggaag   780

atgtccaggccctggggtgaggctgggagctcatggatggaggagggggggtcccagttt   840
             t3
gaccttgagtctcccccttccagCGGGTGAAACAGTGTGTGGGGACAGTCACCCTGGATCA   900
                       R  V  K  Q  C  V  G  T  V  T  L  D  Q
GATCAAGGACCCGCTCGACATCACCTGCAATGAGgtgagtggcccccttattggtgtcaag   960
 I  K  D  P  L  D  I  T  C  N  E
ttgctaatgggttggtgtggggaactccttgggagtgttacccgctgccccatccagggc  1020

gtggaaaggccctcctaccccggcccttccctcacctcggccccagggctccaggtctgg  1080

ctctgtcatccttagggccgcggttccctcaatggggtcccccctcgtatttgtcagaa  1140
                                              g3.5
aggcacatttcaggccccaccccgaccctctgaatcacactcttgggtggagcccagcct  1200

tgtctcttctcccaagatcccagcgggttcttcctgtgctgtcggctgagaggcagtgac  1260

cggactaatggacttgcaggccctgctcctggccagctttgcggggctgggtttgggacc  1320

ctggcaaggccccagccatctctgggcctgagtccacttatgtgtctgtgggggattcca  1381
                                          g3.5
                                          t5
ccacgtgctccaaaggtcacagccagaggtggaccagggccccaagcctcttactgtttc  1440
```

Figur 8

```
cccattcagggatttttctagtctggagggagggttcttgtcttgacccttggccagacc    1500
                                          G3
ccacccgaaacctgtttctcttggtcacagGTTCAAGGTGTCAGGGGAGGTCGCCTGTGC    1560
                              V  Q  G  V  R  G  G  R  L  C
                                          G3


             C5                      T5
TATTGTAGGCGTAGGTTCTGCGTCTGTGTCGGACGAGGATGACGGTTGCGACGGCAGGCT    1620
 Y  C  R  R  R  F  C  V  C  V  G  R  G ***
             P5
TTCCCTCCCCCAATTTTCCCGGGGCCAGGTTTCCGTCCCCCAATTTTTCCGCCTCCACCT    1680

TTCCGGCCCGCACCATTCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAGGATTTGCAGG    1740
                     C3,5
CAACTCACCCAGAAGGCCTTTCGGTACATTAAAATCCCAGCAAGGAGACCTAAGCATCTG    1800

CTTTGCCCAGGCCCGCATCTGTCAAATAAATTCTTGTGAAACC                     1843
```

Figure 8.
Sequences of Protegrin Genes
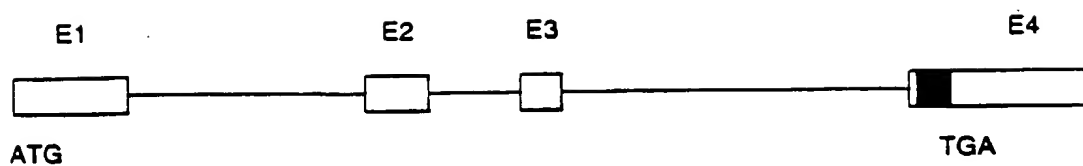
Figure 9.
The organization of Protegrin
Genes
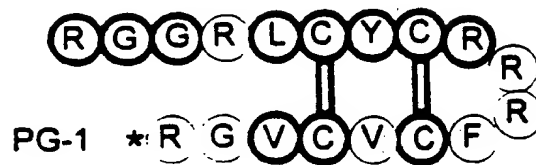
Figure 10

Activity of sPG-1 and sPG-5 against *E. coli* ML-35p
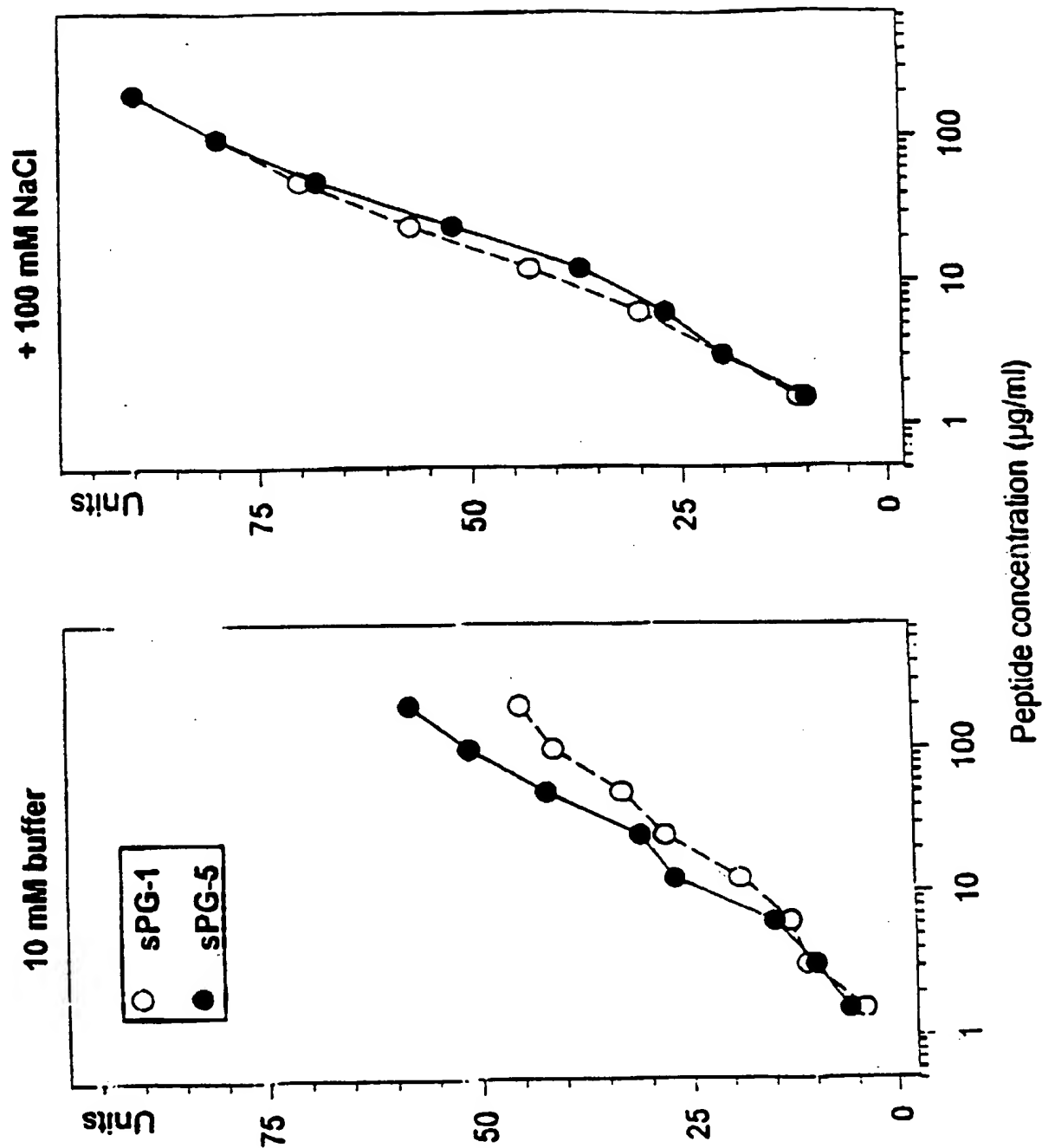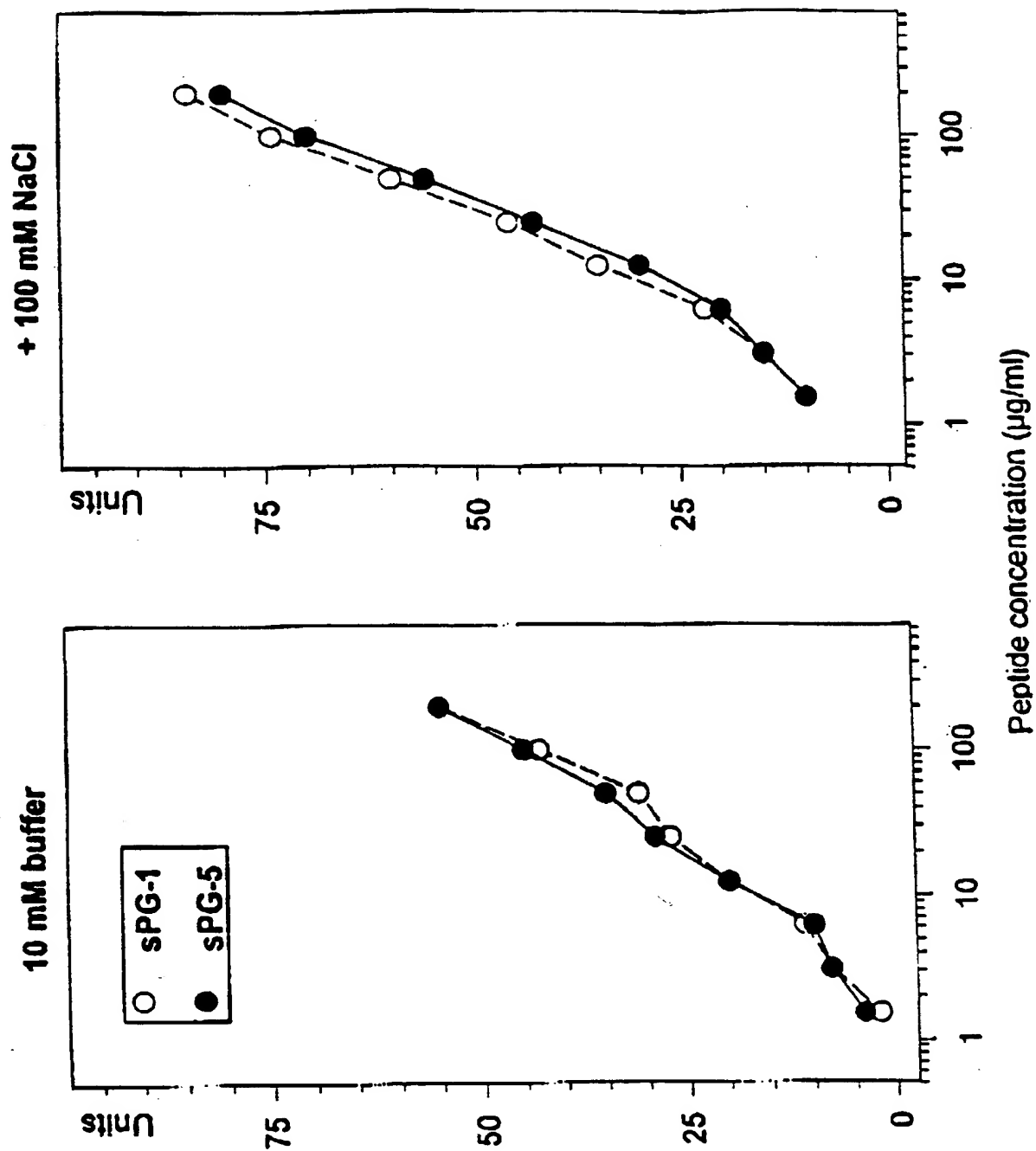
Figure 11a

end1494c.spw

Activity of sPG-1 and sPG-5 against *L. monocytogenes*

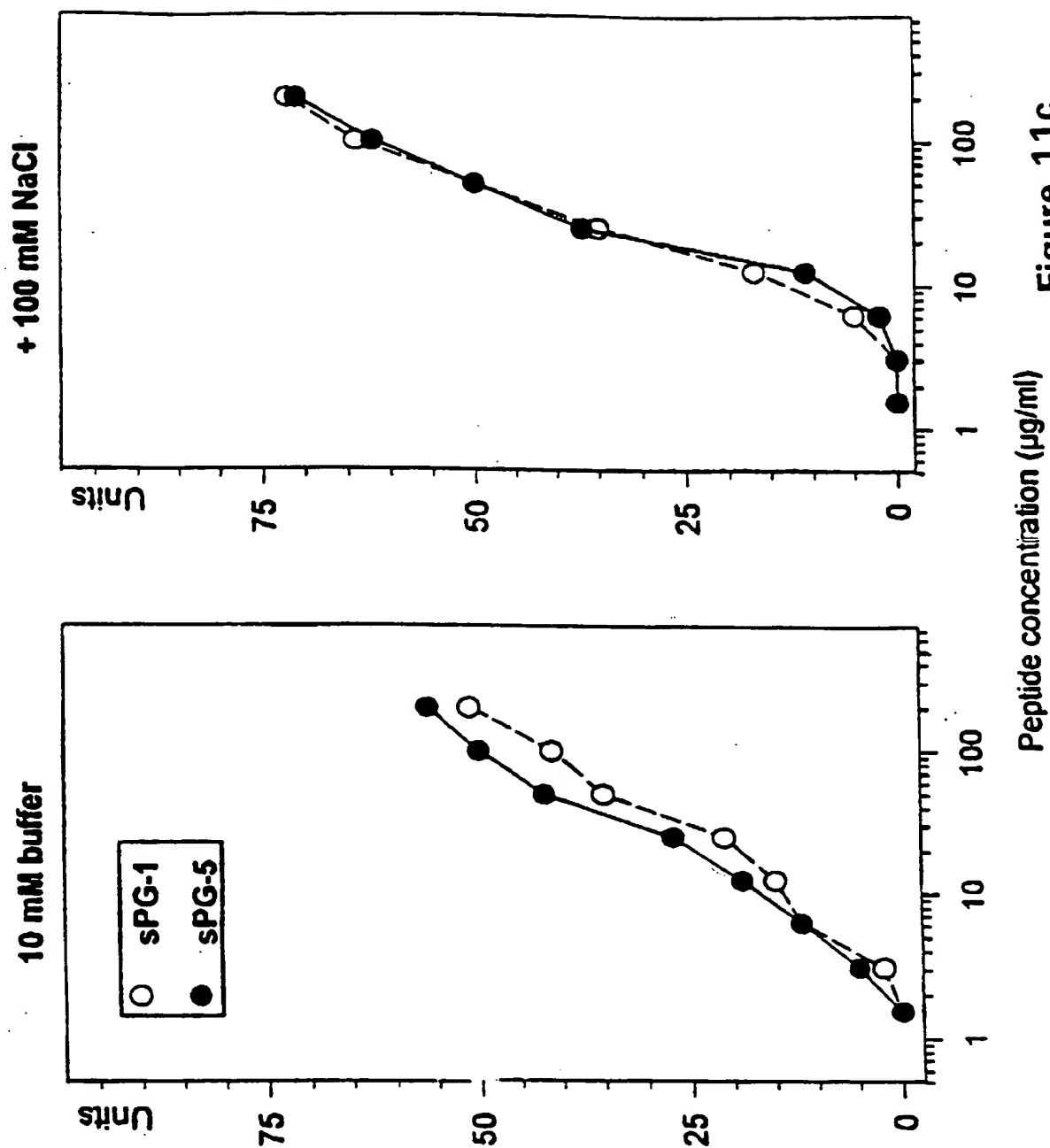Fig 11B

Activity of sPG-1 and sPG-5 against *C. albicans*

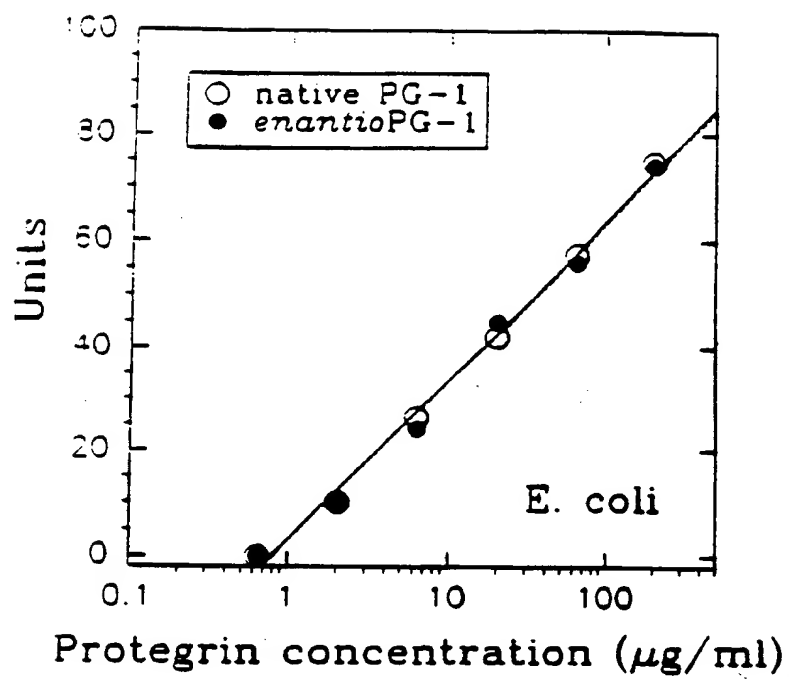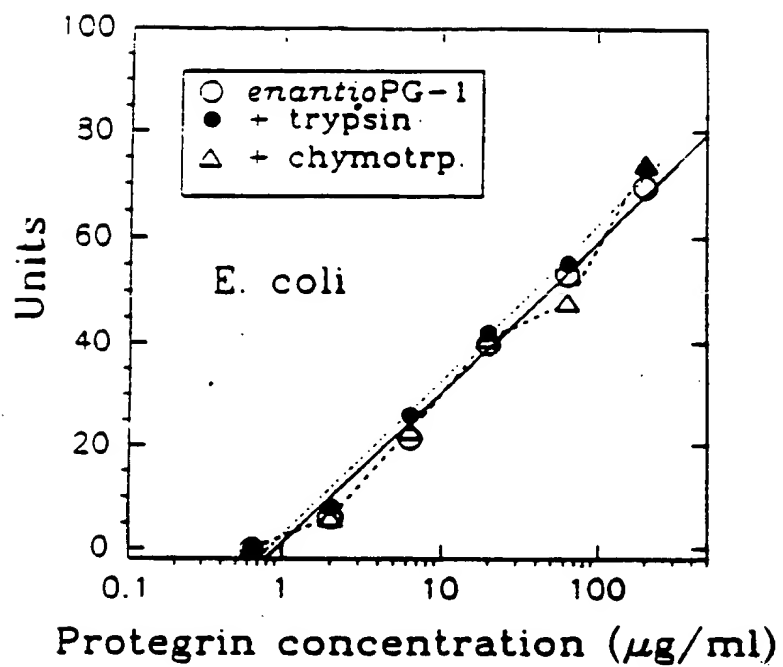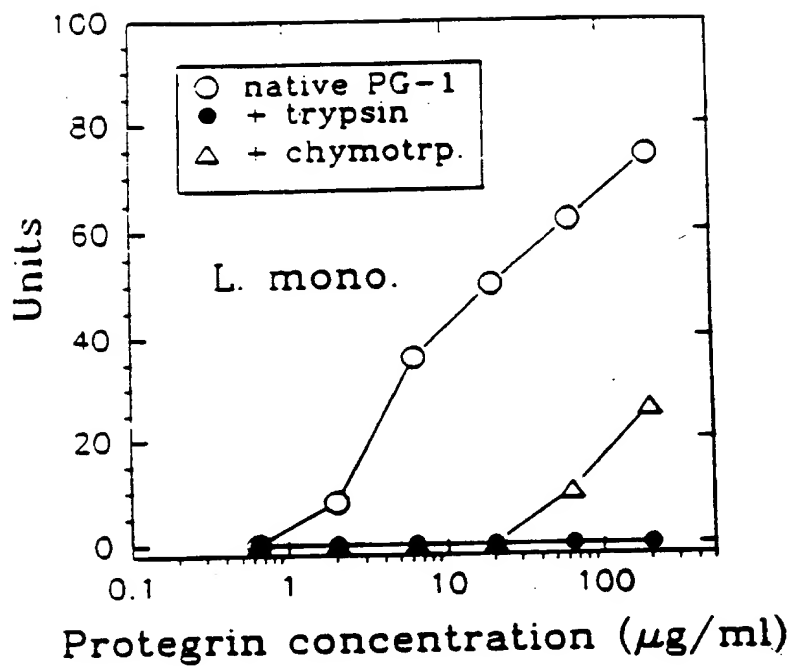Figure 11c

Figure 12a

Figure 12b

Figure 12c
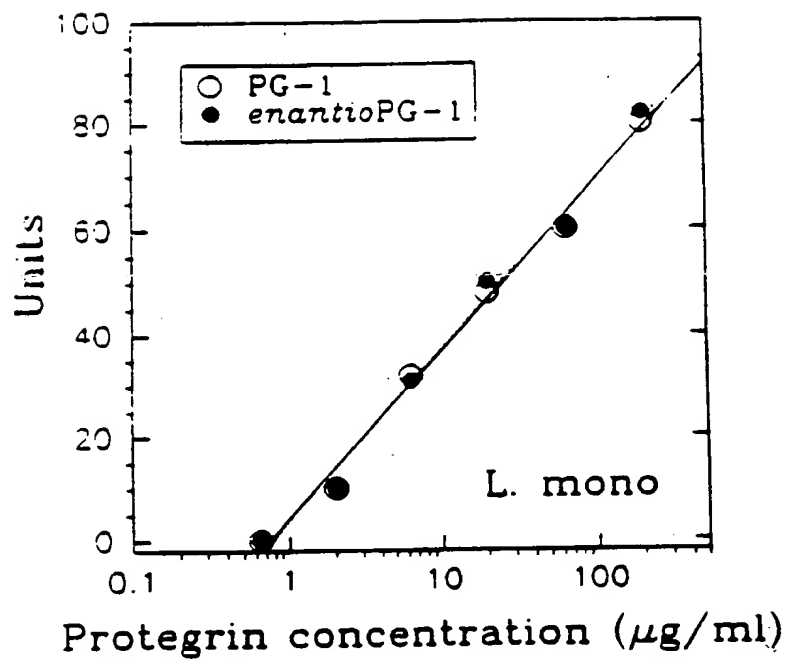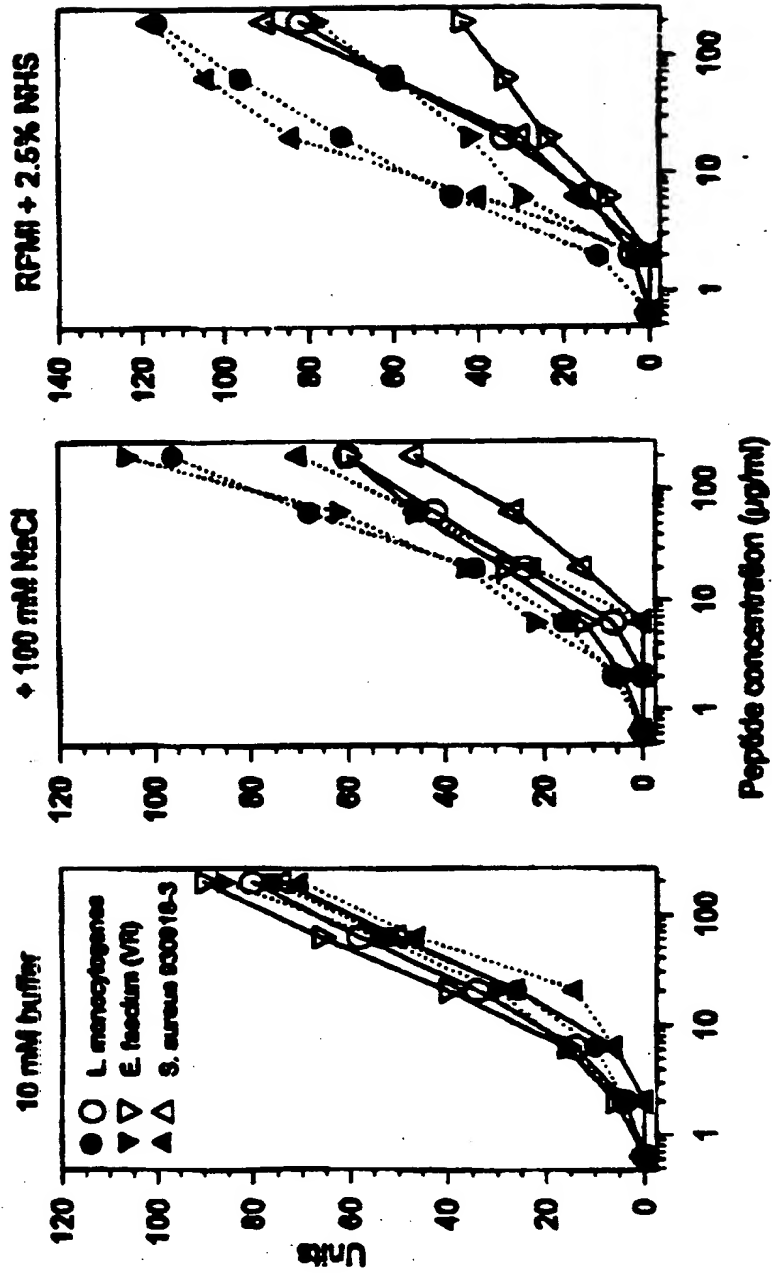
Figure 12d

Figure 13

open symbols = kite, closed symbols = bullet

Figure 14

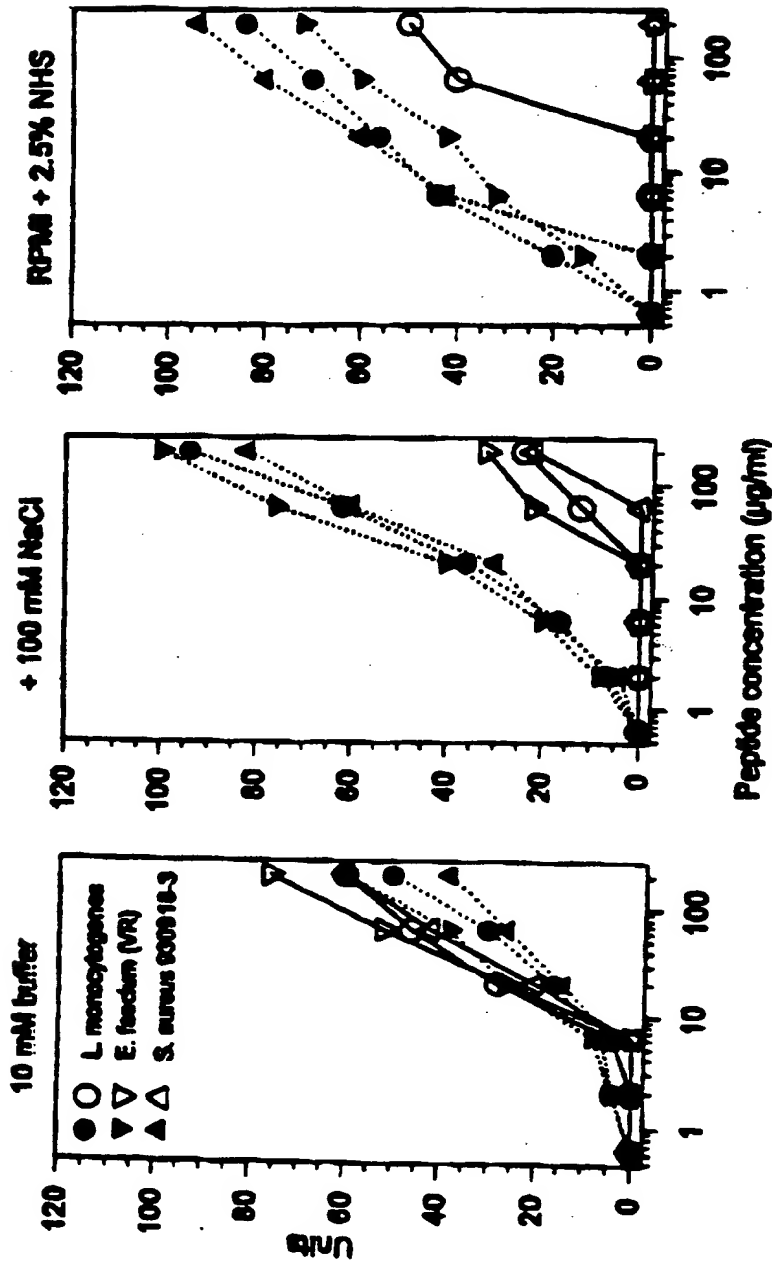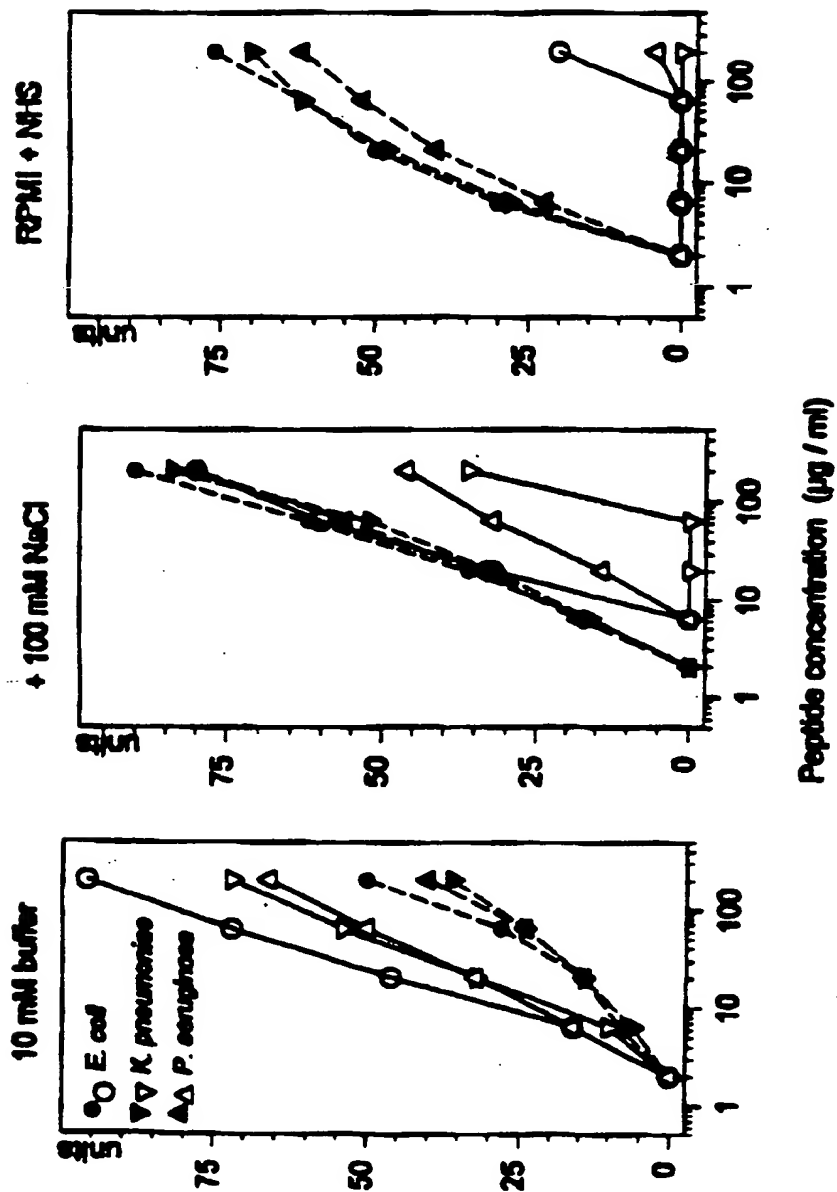Activity of mono-disulfide forms of protegrin PG-1 against Gram-negative bacteria
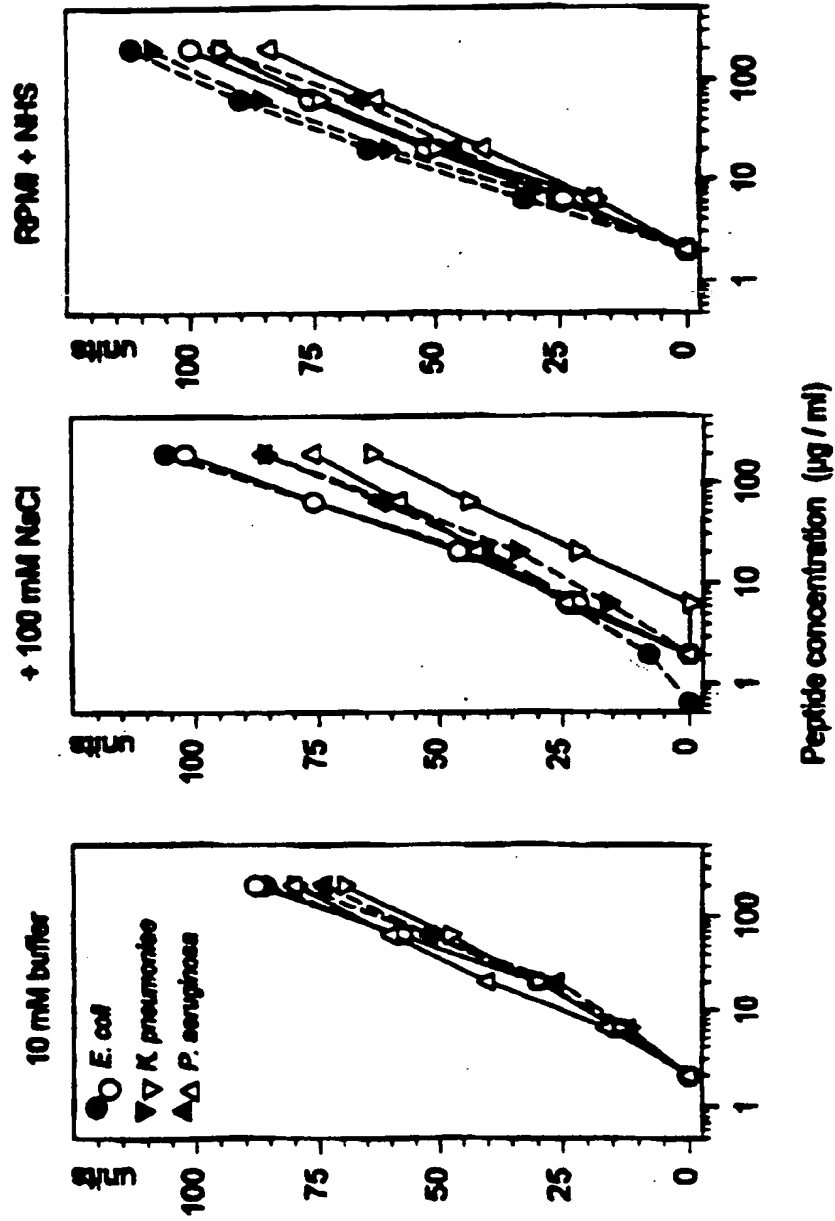
Activity of linearized and native protegrin against Gram-positive bacteria

Figure 15

open symbols = linearized, closed symbols = native

Figure 16

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/00, 7/08; A61K 38/10; C12N 15/12, 15/74

US CL :514/12, 13; 435/69.1, 320.1; 530/324, 325, 326; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 13; 435/69.1, 320.1; 530/324, 325, 326; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOKRYAKOV et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Letters. July 1993, Volume 327, Number 2, pages 231-236, see entire document.	1, 2, 5, 6
A	WO 89/11291 A1 (INVITRON CORPORATION) 30 November 1989, pages 7-12, 14 and 15.	1, 2, 5, 6
A	EP 0 545 730 A1 (PIONEER HI-BRED INTERNATIONAL, INC.) 09 June 1993, pages 5-8.	1
Y	WO 93/24139 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 09 December 1993, see pages 17-22.	1 and 2

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 SEPTEMBER 1996	**1 1 OCT 1996**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	William W. Moore
Facsimil No. (703) 305-3230	Telephone No. (703) 308-0196

F rm PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94/21672 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 29 September 1994, see pages 6-20, 28, 29 and 34-36.	1, 2, 5, 6
Y	WO 95/10534 A1 (SEIKAGAKU CORPORATION) 20 April 1995, see pages 4-30.	1-3, 5, 6
X	MIRGORODSKAYA et al. Primary structure of three cationic peptides from porcine neutrophils. FEBS Letters, September 1993, Volume 330, Number 3, pages 339-342, see page 341.	1, 2, 5, 6
X	STORICI et al. A novel cDNA sequence encoding a pig leukocyte antimicrobial peptide with a cathelin-like pro-sequence. Biochemical and Biophysical Research Communications. 15 November 1993, Volume 196, No. 3, pages 1363-1368, see pages 1364-1364 and Figures 1 and 2.	1, 2, 5, 6
X -- Y	ZHAO et al. Identification of a new member of the protegrin family by cDNA cloning. FEBS Letters. June 1994, Volume 346, pages 285-288, see pages 286-288 and Figures 1-4.	1, 2, 6 --------- 5
X	WO 95/03325 A1 (UNIVERSITY OF CALIFORNIA, LOS ANGELES) 02 February 1995, see entire document.	1-6
A	MATSUZAKI et al. Role of Disulfide Linkages in Tachyplesin-Lipid Interactions. Biochemistry. November 1993, Volume 32, pages 11704-11710.	1-5
A	TAMAMURA et al. A comparative study of the solution structures of tachyplesin I and a novel anti-HIV synthetic peptide, T22 ([Tyr5,12, Lys7]-polyphemusin II), determined by nuclear magnetic resonance. Biochimica et Biophysica Acta. May 1993, Volume 1163, pages 209-216.	1, 2 and 5
A	TAMAMURA et al. Antimicrobial Activity and Conformation of Tachyplesin I and Its Analogs. Chemical and Pharmaceutical Bulletin. May 1993, Volume 41, No. 5, pages 978-980.	1, 2, 4, 5

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet) |

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-16
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Pr test   ☐ The additional search fees were accompanied by the applicant's protest.
   ☐ No protest accompanied the payment of additional search fees.

F rm PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

A-GeneSeq23, PIR47, Swiss-Prot32 & issued U.S. Patent protein databases were searched with SEQ IDs NOs:2, 4, 6, 8, and 10-67 of the latter priority document; DIALOG databases Medline, CA Seach, A_.icola, Biosis Previews, Derwent Biotech Abs., Current Biotech Abs. and Derwent World Patent Index were searched with terms defensin, corticostatin, protegrin, tachyplesin, cryptdin, cecropin, magainin and antimicrobial peptide

CORRECTED VERSION

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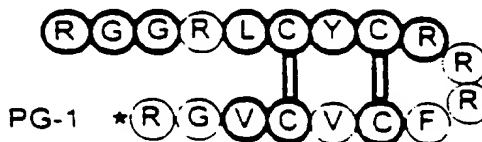
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Published
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(54) Title: PROTEGRINS

	1 2 3	4	5 6 7 8 9	10 11 12	13	14	15 16	17 18
PG-1	RGG	R	LCYCR	RRF	C	V	CV	GR*
PG-2	RGG	R	LCYCR	RRF	C	I	CV	
PG-3	RGG	G	LCYCR	RRF	C	V	CV	GR*
PG-4	RGG	R	LCYCR	GWI	C	F	CV	GR*
PG-5	RGG	R	LCYCR	PRF	C	V	CV	GR*

(57) Abstract

Cationic antimicrobial and virus-neutralizing peptides having 16 to 18 amino acids and comprising 0-4 cysteines are provided as five native protegrins isolated from porcine leukocyte granules having two cystine bridges or as various protegrin analogs having no, or a single, cystine bridge. Native protegrins have, and analogs may have, carboxyl-terminal amidation and analogs may optionally be prepared in amino-terminal acylated and/or cysteine-stabilized and/or carboxyl-terminal esterified forms. Any of the 1-4 native cysteines may be replaced with a hydrophobic or a small amino acid and various substituents are disclosed for the remaining 12-16 positions. Recombinant host cells and methods for production are disclosed, as well as pharmaceutical compositions, compositions for agricultural application, and methods for bacteriostatic, virus-neutralizing, and endotoxin-inactivating use of native protegrins and their analogs.

PROTEGRINS

Technical Field

The invention relates to the field of antibiotic
peptides. In particular, the invention concerns short
10 peptides, some of which are isolated from porcine
leukocytes, that have a wide range of antimicrobial
activities.

Background Art

15   One of the defense mechanisms against infection by both
animals and plants is the production of peptides that have
antimicrobial and antiviral activity. Various classes of
these peptides have been isolated from tissues both of
plants and animals. One well known class of such peptides
20 is the tachyplesins which were first isolated from the
hemocytes of the horseshoe crab as described by Nakamura, T.
et al. J Biol Chem (1988) 263:16709-16713. This article
described the initial tachyplesin isolated, Tachyplesin I,
from the Japanese species. Tachyplesin I is a 17-amino acid
25 amidated peptide containing four cysteine residues providing
two intramolecular cystine bonds. A later article by this
group, Miyata, T. et al. J Biochem (1989) 106:663-668,
reports the isolation of a second tachyplesin, Tachyplesin
II, consisting of 17 residues amidated at the C-terminus,
30 also containing four cysteine residues and two
intramolecular disulfide bonds. Two additional 18-mers,
called polyphemusins, highly homologous to Tachyplesin II
and containing the same positions for the four cysteine
residues, were also isolated from the American horseshoe
35 crab. Polyphemusin I and Polyphemusin II differ from each
other only in the replacement of one arginine residue by a
lysine. All of the peptides were described as having
antifungal and antibacterial activity. A later article by

Murakami, T. *et al.* Chemotherapy (1991) 37:327-334,
describes the antiviral activity of the tachyplesins with
respect to vesicular stomatitis virus; Herpes Simplex Virus
I & II, Adenovirus I, Reovirus II and Poliovirus I were
5 resistant to inactivation by Tachyplesin I. Morimoto, M. *et
al.* Chemotherapy (1991) 37:206-211, found that Tachyplesin I
was inhibitory to Human Immunodeficiency Virus. This anti-
HIV activity was found also to be possessed by a synthetic
analog of Polyphemusin II as described by Nakashima, H. *et
10 al.* Antimicrobial Agents and Chemotherapy (1992) 1249-1255.
Antiviral peptides have also been found in rabbit leukocytes
as reported by Lehrer, R.I. *et al.* J Virol (1985) 54:467-
472.

    Other important classes of cysteine-containing
15 antimicrobial peptides include the defensins, β-defensins
and insect defensins. The defensins are somewhat longer
peptides characterized by six invariant cysteines and three
intramolecular cystine disulfide bonds. Defensins were
described by Lehrer, R.I. *et al.* Cell (1991) 64:229-230;
20 Lehrer, R.I. *et al.* Ann Rev Immunol (1993) 11:105-128. A
review of mammalian-derived defensins by Lehrer, R.I. *et al.*
is found in Annual Review Immunol (1993) 11:105-128; three
patents have issued on the defensins: U.S. 4,705,777; U.S.
4,659,692; and U.S. 4,543,252. Defensins have been found in
25 the polymorphonucleated neutrophils (PMN) of humans and of
several other animals, as well as in rabbit pulmonary
alveolar macrophages, and in murine small intestinal
epithelial (Paneth) cells and in corresponding cells in
humans.

30   β-Defensins are found in bovine respiratory epithelial
cells, bovine granulocytes and avian leukocytes. See
Selsted, M.E. *et al.* J Biol Chem (1993) 288:6641-6648 and
Diamond, G. *et al.* Proc Natl Acad Sci (USA) (1991) 88:3952-
3958. Insect defensins have been reported by Lambert, J. *et
35 al.* Proc Natl Acad Sci (USA) (1989) 88:262-265.

    Antifungal and antibacterial peptides and proteins have
also been found in plants (Broekaert, W.F. *et al.*

Biochemistry (1992) 31:4308-4314) as reviewed by Cornelissen, B.J.C. *et al*. Plant Physiol (1993) 101:709-712. Expression systems for the production of such peptides have been used to transform plants to protect the plants against

5 such infection as described, for example, by Haln, R. *et al*. Nature (1993) 361:153-156.

The present invention provides a new class of antimicrobial and antiviral peptides, designated "protegrins" herein, representative members of which have

10 been isolated from porcine leukocytes. These peptides are useful as antibacterial antiviral and antifungal agents in both plants and animals.

The isolation of the protegrin peptides of the invention was reported by the present applicants in a paper

15 by Kokryakov, V.N. *et al*. FEBS (1993) 337:231-236 (July issue). A later publication of this group described the presence of a new protegrin, whose sequence, and that of its precursor, was deduced from its isolated cDNA clone. Zhao, C et al, FEBS Letters (1994) 346:285-288. An additional

20 paper disclosing cationic peptides from porcine neutrophils was published by Mirgorodskaya, O.A. *et al*. FEBS (1993) 330:339-342 (September issue). Storici, P. *et al*. Biochem Biophys Res Comm (1993) 196:1363-1367, report the recovery of a DNA sequence which encodes a pig leukocyte

25 antimicrobial peptide with a cathelin-like prosequence. The peptide is reported to be one of the protegrins disclosed hereinbelow. Additional publications related to protegrins are Harwig, S.S.L., et al. J. Peptide Sci. (1995) in press; and Zhao, C., et al. FEBS-MS MB-283 (1995) in press.

30 The protegrins of the invention have also been found to bind to endotoxins -- i.e., the lipopolysaccharide (LPS) compositions derived from gram-negative bacteria which are believed responsible for gram-negative sepsis. This type of sepsis is an extremely common condition and is often fatal.

35 Others have attempted to design and study proteins which bind LPS/endotoxin, and illustrative reports of these attempts appear in Rustici, A. *et al*. Science (1993) 259:361-364; Matsuzaki, K. *et al*. Biochemistry (1993)

32:11704-11710; Hoess, A. *et al.* EMBO J (1993) 12:3351-3356;
and Elsbach, P. *et al.* Current Opinion in Immunology (1993)
5:103-107.   The protegrins of the present invention provide
additional compounds which are capable of inactivating of

5   LPS and ameliorating its effects.

        In addition to the foregoing, the protegrins of the
invention are effective in inhibiting the growth of
organisms that are associated with sexually transmitted
diseases.   It is estimated that 14 million people world-wide

10   are infected with HIV and that millions of women sustain
pelvic inflammatory disease each year.   *Chlamydia
trachomatis* and *Neisseria gonorrhoeae* cause over half of
this inflammatory disease although *E. coli*, *Mycoplasma
hominis* and other infectious microorganisms can also be

15   responsible.   Pathogens include viral, bacterial, fungal and
protozoan pathogens.   It is especially important that the
antibiotics used to combat these infections be effective
under physiological conditions.   The protegrins of the
present invention offer these properties.

20

Disclosure of the Invention

        In one embodiment, the invention is directed to
peptides of 16-18 amino acid residues characterized by four
invariant cysteines and either by a characteristic pattern

25   of basic and hydrophobic amino acids and/or being isolatable
from animal leukocytes using the method of the invention.
In a second embodiment, the invention is directed to the
above peptides wherein 1-4 of these cysteines is replaced by
a hydrophobic or small amino acid.   All of these peptides

30   can be produced synthetically and some can be produced
recombinantly or can be isolated from their native sources
and purified for use as preservatives or in pharmaceutical
compositions in treating or preventing infection in animals.
Alternatively, the peptides can be formulated into

35   compositions which can be applied to plants to protect them
against viral or microbial infection.   In still another
approach, the DNA encoding the peptides can be expressed *in
situ*, in animals or preferably in plants, to combat

infections. The peptides are also useful as standards in antimicrobial assays and in binding endotoxins.

Accordingly, in one aspect, the invention is directed to a purified and isolated or recombinantly produced
5 compound of the formula

$$A_1\text{-}A_2\text{-}A_3\text{-}A_4\text{-}A_5\text{-}C_6\text{-}A_7\text{-}C_7\text{-}A_9\text{-}A_{10}\text{-}A_{11}\text{-}A_{12}\text{-}C_{13}\text{-}A_{14}\text{-}C_{15}\text{-}A_{16}\text{-}(A_{17}\text{-}A_{18}) \quad (1)$$

and the N-terminal acylated and/or C-terminal amidated
10 or esterified forms thereof, which is either in the optionally -SH stabilized linear or in a cystine-bridged form

wherein A_1 is a basic amino acid;

each of A_2 and A_3 is independently a small amino acid;
15   each of A_5, A_7, A_{14} is independently a hydrophobic amino acid;

A_4 is a basic or a small amino acid;

each of A_9, A_{12} and A_{16} is independently a basic, a hydrophobic, a neutral/polar or a small amino acid;
20   each of A_{10} and A_{11} is independently a basic, a neutral/polar, a hydrophobic or a small amino acid or is proline;

A_{17} is not present or, if present, is a basic, a neutral/polar, a hydrophobic or a small amino acid;
25   A_{18} is not present or, if present, is a basic, a hydrophobic, a neutral/polar or a small amino acid, or a modified form of Formula (1) and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof wherein at least one of the 4 cysteines is
30 independently replaced by a hydrophobic amino acid or a small amino acid;

with the proviso that the compound of Formula (1) must have a charge of +3 or greater.

In still other aspects, the invention is directed to
35 recombinant materials useful for the production of the peptides of the invention as well as plants or animals modified to contain expression systems for the production of these peptides. The invention is also directed to

pharmaceutical compositions and compositions for application
to plants containing the peptides of the invention as active
ingredients or compositions which contain expression systems
for production of the peptides or for *in situ* expression of
5 the nucleotide sequence encoding these peptides. The
invention is also directed to methods to prepare the
invention peptides synthetically, to antibodies specific for
these peptides, and to the use of the peptides as
preservatives.

10   In other aspects, the invention is directed to the use
of the compounds of the invention as standards in
antimicrobial assays. The compounds many also be used as
antimicrobials in solutions useful in eye care, such as
contact lens solutions, and in topical or other
15 pharmaceutical compositions for treatment of sexually
transmitted diseases (STDs). The invention is also directed
to use of the invention compounds as preservatives for foods
or other perishables. As the invention peptides can
inactivate endotoxin, the invention is also directed to a
20 method to inactivate endotoxins using the compounds of the
invention and to treat gram-negative sepsis by taking
advantage of this property.

Brief Description of the Drawings

25   Figure 1 shows the elution pattern of a concentrate of
the ultrafiltrate of porcine leukocytes applied to a Biogel
P10 column.

Figure 2 shows the antibacterial activity of the P10
fractions obtained from elution of the column described in
30 Figure 1.

Figure 3 shows an elution pattern obtained when
fractions 76-78 from the Biogel P10 column of Figure 1 is
applied to HPLC.

Figure 4 shows the antimicrobial activity of the
35 purified porcine protegrins of the invention:

Figure 4a shows antibacterial activity against *E. Coli*;

Figure 4b shows antibacterial activity against *Listeria
monocytogenes*;

Figure 4c shows antifungal activity against *Candida albicans*;

Figure 4d shows antibacterial activity against *S. aureus*.

Figure 4e shows antibacterial activity against *K. pneumoneae*.

Figure 5 shows the effect of various test conditions on antimicrobial activity:

Figure 5a shows activity against *Candida albicans* in 100 μM NaCl;

Figure 5b shows activity against *E. Coli* in 100 μM NaCl;

Figure 5c shows activity against *Candida albicans* in 90% fetal calf serum.

Figure 6 shows the antimicrobial activity of the linear forms of the protegrins under various test conditions:

Figure 6a shows the activity against *E. coli* in 10 mM phosphate-citrate buffer, pH 6.5;

Figure 6b shows the activity against *E. coli* in the same buffer with 100 mM NaCl;

Figure 6c shows the activity against *L. monocytogenes* in the buffer of Figures 6a-6b;

Figure 6d shows the activity against *L. monocytogenes* in the same buffer with the addition of 100 mM NaCl;

Figure 6e shows the activity against *C. albicans* in the presence of 10 mM phosphate; and

Figure 6f shows the activity against *C. albicans* in the presence of 10 mM phosphate plus 100 mM NaCl.

Figure 7 shows a composite of cDNA encoding the precursors of PG-1, PG-2, PG-3 and PG-4.

Figure 8 shows the nucleotide sequence and the deduced amino acid sequence of the genomic DNA encoding the precursor protein for the antimicrobial compounds of the invention PG-1, PG-3, and PG-5.

Figure 9 shows the organization of the protegrin genomic DNA.

- 8 -

Figure 10 shows the amino acid sequences of the protegrins PG-1 to PG-5.

Figures 11a-11c show the antimicrobial activity of synthetically prepared PG-5 as compared to that of
5   synthetically prepared PG-1.

Figures 12a-12d show the effects of various protegrins against various target microbes.

Figure 13 shows a graphical representation of the effects of the kite and bullet forms of PG-1 against gram
10 positive bacteria.

Figure 14 shows a graphical representation of the effects of the kite and bullet forms of PG-1 against gram negative bacteria.

Figure 15 is a graphical representation of the
15 antimicrobial activity of the snake form of PG-1 against gram positive bacteria.

Figure 16 is a graphical representation of the antimicrobial activity of the snake form of PG-1 against gram negative bacteria.

20

Modes of Carrying Out the Invention

The peptides of the invention are described by the formula:

25 $A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18})$ (1)

and its defined modified forms. Those peptides which occur in nature must be in purified and isolated form or prepared recombinantly.

30   The designation A_n in each case represents an amino acid at the specified position in the peptide. As A_{17} and A_{18} may or may not be present, the peptides of the invention contain either 16, 17 or 18 amino acids. The positions of the cysteine residues, shown as C in Formula (1), are
35 invariant in the peptides of the invention; however, in the modified forms of the peptides of Formula (1), also included within the scope of the invention, at least one of 1-4 of

these cysteines may be replaced by a hydrophobic or small amino acid.

The amino terminus of the peptide may be in the free amino form or may be acylated by a group of the formula
5   RCO-, wherein R represents a hydrocarbyl group of 1-6C. The hydrocarbyl group is saturated or unsaturated and is typically, for example, methyl, ethyl, i-propyl, t-butyl, n-pentyl, cyclohexyl, cyclohexene-2-yl, hexene-3-yl, hexyne-4-yl, and the like.
10   The C-terminus of the peptides of the invention may be in the form of the underivatized carboxyl group, either as the free acid or an acceptable salt, such as the potassium, sodium, calcium, magnesium, or other salt of an inorganic ion or of an organic ion such as caffeine. The carboxyl
15   terminus may also be derivatized by formation of an ester with an alcohol of the formula ROH, or may be amidated by an amine of the formula NH_3, or RNH_2, or R_2NH, wherein each R is independently hydrocarbyl of 1-6C as defined above. Amidated forms of the peptides wherein the C-terminus has
20   the formula $CONH_2$ are preferred.

As the peptides of the invention contain substantial numbers of basic amino acids, the peptides of the invention may be supplied in the form of the acid addition salts. Typical acid addition salts include those of inorganic ions
25   such as chloride, bromide, iodide, fluoride or the like, sulfate, nitrate, or phosphate, or may be salts of organic anions such as acetate, formate, benzoate and the like. The acceptability of each of such salts is dependent on the intended use, as is commonly understood.
30   The peptides of the invention that contain at least two cysteines may be in straight-chain or cyclic form. The straight-chain forms are convertible to the cyclic forms, and vice versa. Methods for forming disulfide bonds to create the cyclic peptides are well known in the art, as are
35   methods to reduce disulfides to form the linear compounds. The linear compounds can be stabilized by addition of a suitable alkylating agent such as iodoacetamide.

The cyclic forms are the result of the formation of cystine linkages among all or some of the four invariant cysteine residues. Cyclic forms of the invention include all possible permutations of cystine bond formation; if the cysteines are numbered in order of their occurrence starting at the N-terminus as C_6, C_8, C_{13} and C_{15}, these permutations include:

C_6-C_8;

C_6-C_{13};

C_6-C_{15};

C_8-C_{13};

C_8-C_{15};

C_{13}-C_{15};

C_6-C_8, C_{13}-C_{15};

C_6-C_{13}, C_8-C_{15}; and

C_6-C_{15}, C_8-C_{13}.

In the modified forms of the peptides, where 1-4 cysteines are replaced, similar permutations are available when 2-3 cysteines are present.

The native forms of the protegrins contain two cystine bonds are between the cysteine at position 6 and the cysteine at position 15 and the other between the cysteine at position 8 and the cysteine at position 13. Accordingly, in those embodiments having two cystine linkages, the C_6-C_{15}, C_8-C_{13} form is preferred. However, it has been found by the present applicants that forms of the protegrins containing only one cystine linkage are active and easily prepared. Preferred among embodiments having only one cystine linkage are those represented by C_6-C_{15} alone and by C_8-C_{13} alone.

Forms containing a C_6-C_{15} cystine as the only cystine linkage are generally designated "bullet" forms of the protegrins; those wherein the sole cystine is C_8-C_{13} are designated the "kite" forms. The bullet and kite forms can most conveniently be made by replacing the cystines at the positions not to be linked by cystine with a neutral amino acid, preferably a small amino acid such as glycine, serine, alanine or threonine and less preferably a neutral polar amino acid such as asparagine or glutamine. Thus, in

embodiments of the bullet form, each of C_8 and C_{13} is
independently alanine, serine, threonine or glycine,
preferably both are alanine. Conversely, in the kite form,
C_6 and C_{15} are thus replaced.

5   As the linearalized forms of the native cyclic peptides
have valuable activities, even when chemically stabilized to
preserve the sulfhydryl form of cysteine for example, by
reaction with iodoacetamide, the compounds of the invention
also include linearalized forms which are stabilized with

10   suitable reagents. As defined herein, "SH-stabilized" forms
of the peptides of the invention contain sulfhydryl groups
reacted with standard reagents to prevent reformation into
disulfide linkages.

      An alternative approach to providing linear forms of

15   the protegrins of the invention comprises use of the
modified form of the peptides where cysteine residues are
replaced by amino acids which do not form cystine linkages.
In this instance, too, all 4 (or at least 3) of the cystines
at positions 6, 8, 13, and 15 are replaced by polar neutral

20   or small amino acids as listed above. It is preferred that
all 4 cysteine residues be replaced in order to minimize the
likelihood of intermolecular bonding.

      The amino acids denoted by A_n may be those encoded by
the gene or analogs thereof, and may also be the D-isomers

25   thereof. One preferred embodiment of the peptides of the
invention is that form wherein all of the residues are in
the D-configuration thus conferring resistance to protease
activity while retaining antimicrobial or antiviral
properties. The resulting protegrins are themselves

30   enantiomers of the native L-amino acid-containing forms.

      The amino acid notations used herein are conventional
and are as follows:

Amin Acid	One-Letter Symb l	Three-Letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

The amino acids not encoded genetically are abbreviated as indicated in the discussion below.

In the specific peptides shown in the present
5   application, the L-form of any amino acid residue having an optical isomer is intended unless the D-form is expressly indicated by a dagger superscript (†).

The compounds of the invention are peptides which are partially defined in terms of amino acid residues of
10   designated classes. Amino acid residues can be generally subclassified into major subclasses as follows:

Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the
15   conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface

positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Hydrophobic: The residues are not charged at
5   physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

Neutral/polar: The residues are not charged at
10   physiological pH, but the residue is not sufficiently repelled by aqueous solutions so that it would seek inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

This description also characterizes certain amino acids
15   as "small" since their side chains are not sufficiently large, even if polar groups are lacking, to confer hydrophobicity.   "Small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not.

20   It is understood, of course, that in a statistical collection of individual residue molecules some molecules will be charged, and some not, and there will be an attraction for or repulsion from an aqueous medium to a greater or lesser extent.   To fit the definition of
25   "charged," a significant percentage (at least approximately 25%) of the individual molecules are charged at physiological pH.   The degree of attraction or repulsion required for classification as polar or nonpolar is arbitrary and, therefore, amino acids specifically
30   contemplated by the invention have been classified as one or the other.   Most amino acids not specifically named can be classified on the basis of known behavior.

Amino acid residues can be further subclassified as cyclic or noncyclic, and aromatic or nonaromatic, self-
35   explanatory classifications with respect to the side-chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon,

provided an additional polar substituent is present; three
or less if not. Small residues are, of course, always
nonaromatic.

For the naturally occurring protein amino acids,
subclassification according to the foregoing scheme is as
follows.

<u>Acidic</u>:   Aspartic acid and Glutamic acid;

<u>Basic</u>:   Noncyclic: Arginine, Lysine;
                Cyclic: Histidine;

<u>Small</u>:   Glycine, Serine, Alanine, Threonine;

<u>Polar/large</u>: Asparagine, Glutamine;

<u>Hydrophobic</u>:   Tyrosine, Valine, Isoleucine, Leucine,
                  Methionine, Phenylalanine, Tryptophan.

The gene-encoded secondary amino acid proline is a
special case due to its known effects on the secondary
conformation of peptide chains, and is not, therefore,
included in a group. Cysteine residues are also not
included in these classifications since their capacity to
form disulfide bonds to provide secondary structure is
critical in the compounds of the present invention.

Certain commonly encountered amino acids, which are not
encoded by the genetic code, include, for example, beta-
alanine (beta-Ala), or other omega-amino acids, such as
3-aminopropionic, 2,3-diaminopropionic (2,3-diaP),
4-aminobutyric and so forth, alpha-aminisobutyric acid
(Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit),
t-butylalanine (t-BuA), t-butylglycine (t-BuG),
N-methylisoleucine (N-MeIle), phenylglycine (Phg), and
cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine
(2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
(Tic); β-2-thienylalanine (Thi); methionine sulfoxide (MSO);

- 15 -

and homoarginine (Har). These also fall conveniently into particular categories.

Based on the above definitions,

Sar, beta-Ala, 2,3-diaP and Aib are small;

t-BuA, t-BuG, N-MeIle, Nle, Mvl, Cha, Phg, Nal, Thi and Tic are hydrophobic;
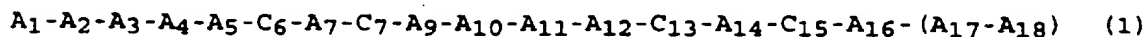
Orn and Har are basic;

Cit, Acetyl Lys, and MSO are neutral/polar.

The various omega-amino acids are classified according to size as small (beta-Ala and 3-aminopropionic) or as large and hydrophobic (all others).

Other amino acid substitutions of those encoded in the gene can also be included in peptide compounds within the scope of the invention and can be classified within this general scheme according to their structure.

In all of the peptides of the invention, one or more amide linkages (-CO-NH-) may optionally be replaced with another linkage which is an isostere such as $-CH_2NH-$, $-CH_2S-$, $-CH_2CH_2$, -CH=CH- (cis and trans), $-COCH_2-$, $-CH(OH)CH_2-$ and $-CH_2SO-$. This replacement can be made by methods known in the art. The following references describe preparation of peptide analogs which include these alternative-linking moieties: Spatola, A.F., _Vega Data_ (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Spatola, A.F., in "Chemistry and Biochemistry of Amino Acids Peptides and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J.S., _Trends Pharm Sci_ (1980) pp. 463-468 (general review); Hudson, D., et al., _Int J Pept Prot Res_ (1979) 14:177-185 $(-CH_2NH-$, $-CH_2CH_2-)$; Spatola, A.F., et al., _Life Sci_ (1986) 38:1243-1249 $(-CH_2-S)$; Hann, M.M., _J Chem Soc Perkin Trans I_ (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G., et al., _J Med Chem_ (1980) 23:1392-1398 $(-COCH_2-)$; Jennings-White, C., et al., _Tetrahedron Lett_ (1982) 23:2533 $(-COCH_2-)$; Szelke, M., et al., European Application EP 45665 (1982) CA:97:39405 (1982) $(-CH(OH)CH_2-)$; Holladay, M.W., et al., _Tetrahedron Lett_ (1983) 24:4401-4404 $(-C(OH)CH_2-)$; and Hruby, V.J., _Life Sci_ (1982) 31:189-199 $(-CH_2-S-)$.

The compounds of Formula (1) are generally defined as

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_7-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \quad (1)$$

5   and the N-terminal acylated and/or C-terminal amidated
or esterified forms thereof, which is either in the
optionally -SH stabilized linear or in a cystine-bridged
form
       wherein A_1 is a basic amino acid;
10   each of A_2 and A_3 is independently a small amino acid;
       each of A_5, A_7, A_{14} is independently a hydrophobic amino
acid;
       A_4 is a basic or a small amino acid;
       each of A_9, A_{12} and A_{16} is independently a basic, a
15   hydrophobic, a neutral/polar or a small amino acid;
       each of A_{10} and A_{11} is independently a basic, a
neutral/polar, a hydrophobic or a small amino acid or is
proline;
       A_{17} is not present or, if present, is a basic, a
20   neutral/polar, a hydrophobic or a small amino acid;
       A_{18} is not present or, if present, is a basic, a
hydrophobic, a neutral/polar or a small amino acid, or a
       modified form of Formula (1) and the N-terminal
acylated and/or C-terminal amidated or esterified forms
25   thereof wherein at least one of the 4 cysteines is
independently replaced by a hydrophobic amino acid or a
small amino acid;
       with the proviso that the compound of Formula (1) must
have a charge of +3 or greater.
30   In preferred embodiments of the compounds of the
invention, each of A_1 and A_9 is independently selected from
the group consisting of R, K and Har; more preferably, both
A_1 and A_9 are R.
       In another class of preferred embodiments, each of A_2
35   and A_3 is independently selected from the group consisting
of G, A, S and T; more preferably, A_2 and A_3 are G.

In another set of preferred embodiments, A_4 is selected from the group consisting of R, K, Har, G, A, S and T; more preferably, A_4 is R or G.

In another set of preferred embodiments, each of A_5, A_{14}
and A_{16} is independently selected independently from the group consisting of I, V, L, Nle and F; preferably I, V, L and F.

In another set of preferred embodiments, each of A_7 and A_{12} is independently selected from the group consisting of I, V, L, W, Y and F; preferably A_7 is Y and A_{12} is I or F.

In another set of preferred embodiments, A_{10} is R, G or P.

In another set of preferred embodiments, A_{11} is R or W.

A_{17}, when present, is preferably G, A, S or T, most preferably G;

A_{18}, when present, is preferably R, K or Har, most preferably R.

As described above, the compounds of Formula (1) are either in cyclic or noncyclic (linearalized) form or may be modified wherein 1-4 of the cysteines is replaced by a small amino acid residue or a hydrophobic residue or a nonpolar large amino acid residue. If the linearalized forms of the compound of Formula (1) are prepared, or if linearalized forms of those modified peptides which contain at least two cysteines are prepared, it is preferred that the sulfhydryl groups be stabilized by addition of a suitable reagent. Preferred embodiments for the hydrophobic amino acid to replace cysteine residues are I, V, L and NLe, preferably I, V or L. Preferred small amino acids to replace the cysteine residues include G, A, S and T, most preferably G. Preferred large polar amino acids are N and Q.

In an alternative embodiment, the peptides of the invention are defined as described by Formula (1), but wherein the definitions of A_n in each case are determined by the isolatability of the peptide from animal leukocytes by the invention method. The invention method comprises the steps of providing an ultrafiltrate of a lysate of animal leukocytes and isolating peptides of 16-18 amino acids.

These peptides can further be defined by the ability of DNA
encoding them to hybridize under stringent conditions to DNA
encoding the peptides exemplified as PG-1, PG-2, PG-3, PG-4
and PG-5 herein.

5   Particularly preferred compounds of the invention are:

Unmodified forms

PG-1:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R

PG-2:   R-G-G-R-L-C-Y-C-R-R-R-F-C-I-C-V

10 PG-3:   R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R

PG-4:   R-G-G-R-L-C-Y-C-R-G-W-I-C-F-C-V-G-R

PG-5:   R-G-G-R-L-C-Y-C-R-P-R-F-C-V-C-V-G-R

         R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V

         K-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V

15   R-G-G-Har-L-C-Y-C-R-R-R-F-C-V-C-V

         R-G-G-Har-L-C-Y-C-Har-R-R-F-C-V-C-V-G-R

         R-G-G-R-V-C-Y-C-R-Har-R-F-C-V-C-V-G-R

         R-G-G-R-L-C-Y-C-R-K-K-W-C-V-C-V-G-R

         R-G-G-R-L-C-Y-C-R-Har-R-Y-C-V-C-V-G-R

20   R-G-S-G-L-C-Y-C-R-R-K-W-C-V-C-V-G-R

         R-A-T-R-I-C-F-C-R-R-R-F-C-V-C-V-G-R

         R-G-G-K-V-C-Y-C-R-Har-R-F-C-V-C-V-G-R

         R-A-T-R-I-C-F-C-R†-R-R-F-C-V-C-V-G-R†

         R-G-G-K-V-C-Y-C-R-Har†-R-F-C-V-C-V-G-R

25 PG-1:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R (all †)

PG-2:   R-G-G-R-L-C-Y-C-R-R-R-F-C-I-C-V (all †)

PG-3:   R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R (all †)

PG-4:   R-G-G-R-L-C-Y-C-R-G-W-I-C-F-C-V-G-R (all †)

PG-5:   R-G-G-R-L-C-Y-C-R-P-R-F-C-V-C-V-G-R

30 PC-39:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-R

PC-41:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G

PC-100: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-Y

PC-101: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-T

PC-102: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-A

35 PC-103: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-L

PC-104: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-I

PC-105: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-F

```
PC-106:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W
PC-108:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R
         R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R
         R-G-G-R-L-C-W-C-R-R-R-F-C-V-C-V-G-R
5        R-G-G-R-L-C-Y-C-R-R-R-W-C-V-C-V-G-R
         R-G-G-R-L-C-Y-C-R-R-R-F-C-W-C-V-G-R
         R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W-G-R
IB-247:  R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH
IB-249:  R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH
10 IB-223:  R-G-G-G-L-C-Y-C-R-R-G-F-C-V-C-F-G-R
IB-224:  R-G-G-G-L-C-Y-C-R-R-P-F-C-V-C-V-G-R
IB-324:  R-G-G-G-L-C-Y-C-R-P-R-F-C-V-C-V-G-R-OH
IB-341:  R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R-OH  (X=NMeG)
IB-342:  R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R  (X=NMeG)
15 IB-384:  R-G-G-R-L-C-Y-C-X-G-R-F-C-V-C-V-G-R  (X=Cit)
IB-398:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R
IB-399:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R-OH
IB-218:  R-G-G-G-L-C-Y-C-F-P-K-F-C-V-C-V-G-R
IB-349:  R-G-G-R-L-C-Y-C-R-X-R-Cha-C-V-C-W-G-R  (X=NMeG)
20 IB-350:  R-G-G-R-W-C-V-C-R-X-R-Cha-C-Y-C-V-G-R  (X=NMeG)
IB-394:  R-G-G-R-W-C-V-C-R-G-R-Cha-C-Y-C-V-G-R
IB-416:  R-G-G-R-L-C-Y-C-R-R-R-F-C-NMeV-C-V-G-R
IB-400:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V
IB-401:  R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-OH
25
```

both the linear and mono- and bicyclic forms thereof, and
including the N-terminal acylated and C-terminal amidated
forms;

30 <u>Modified forms</u>

```
         R-G-G-R-L-V-Y-C-R-R-R-F-C-V-C-V-G-R
         R-G-G-R-L-G-Y-C-R-R-R-F-C-I-C-V
         R-G-G-G-L-C-Y-G-R-R-R-F-C-V-C-V-G-R
         R-G-G-R-L-G-Y-G-R-R-R-F-G-V-C-V
35       K-G-G-R-L-V-Y-V-R-R-R-F-I-V-C-V
         R-G-G-Har-L-C-Y-C-R-R-R-F-C-V-G-V
         R-G-G-Har-L-C-Y-C-Har-R-R-F-C-V-L-V-G-R
         R-G-G-R-V-C-Y-V-R-Har-R-F-L-V-G-V-G-R
```

```
            R-G-G-R-L-C-Y-S-R-K-K-W-C-V-S-V-G-R
            R-G-G-R-L-C-Y-C-R-Har-R-Y-S-V-V-V-G-R
            R-G-S-G-L-S-Y-C-R-R-K-W-G-V-C-V-G-R
            R-A-T-R-I-S-F-S-R-R-R-F-S-V-S-V-G-R
5           R-G-G-K-V-C-Y-G-R-Har-R-F-S-V-C-V-G-R
            R-A-T-R-I-V-F-C-R†-R-R-F-G-V-C-V-G-R†
            R-G-G-K-V-C-Y-L-R-Har†-R-F-L-V-C-V-G-R
            R-G-G-R-I-C-F-L-R-P-R-I-G-V-C-V-G-R
    PC-49:  R-G-G-R-L-C-W-A-R-R-R-F-A-V-C-V-G-R
10  PC-50:  R-G-G-R-L-C-Y-A-R-R-R-W-A-V-C-V-G-R
    PC-52:  R-G-G-R-L-A-W-C-R-R-R-F-C-V-A-V-G-R
    PC-53:  R-G-G-R-L-A-Y-C-R-R-R-F-C-V-A-W-G-R
    PC-55:  R-G-G-R-L-A-W-A-R-R-R-F-A-V-A-V-G-R
    PC-56:  R-G-G-R-L-A-Y-A-R-R-R-W-A-V-A-V-G-R
15  PC-57:  R-G-G-R-L-A-Y-A-R-R-R-F-A-V-A-W-G-R
    IB-214: R-G-G-G-L-C-Y-A-R-G-W-I-A-F-C-V-G-R
    IB-216: R-G-G-G-L-C-Y-A-R-G-F-I-A-V-C-F-G-R
    IB-225: R-G-G-G-L-C-Y-A-R-P-R-F-A-V-C-V-G-R
    IB-226: R-G-G-G-L-C-Y-T-R-P-R-F-T-V-C-V-G-R
20  IB-227: R-G-G-G-L-C-Y-A-R-K-G-F-A-V-C-V-G-R
    IB-288: R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R-OH
    IB-289: R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R
```
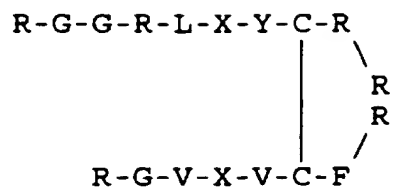
both the linear and cyclic (where possible) forms thereof,
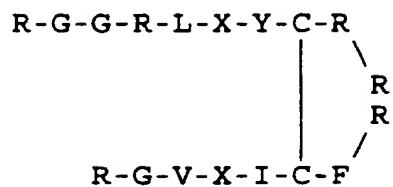25 and including the N-terminal acylated and C-terminal
amidated forms.

        Particularly preferred are compounds wherein a single
cystine bond is formed between C_6 and C_{15} or between C_8 and
C_{13} wherein four compounds having a cystine bond between C_8
30 and C_{13} each of C_6 and C_{15} is independently replaced by "X"
wherein X is a hydrophobic, a small, or a large polar amino
acid. Similarly, where the single cystine bond is between
C_8 and C_{13}, each of C_6 and C_{15} is independently replaced by X
as defined above. Also preferred are the "snake" forms of
35 the compounds of the invention where all 4 cysteines are
replaced by X as defined above. Particularly preferred
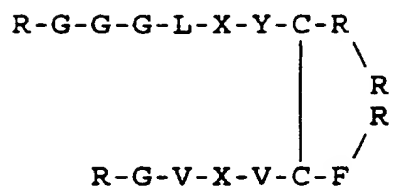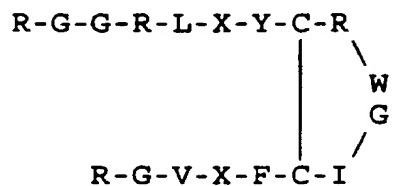embodiments of these compounds of the invention include:

Kite form-1

```
       R-G-G-R-L-X-Y-C-R
                       |   \
5                      |    R
                       |    R
                       |   /
       R-G-V-X-V-C-F
```
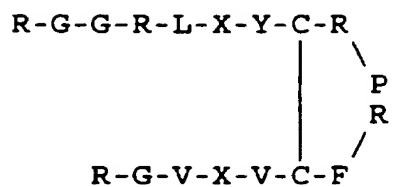
10 Kite form-2

```
       R-G-G-R-L-X-Y-C-R
                       |   \
                       |    R
                       |    R
15                     |   /
       R-G-V-X-I-C-F
```

Kite form-3

```
       R-G-G-G-L-X-Y-C-R
20                     |   \
                       |    R
                       |    R
                       |   /
       R-G-V-X-V-C-F
```

25 Kite form-4

```
       R-G-G-R-L-X-Y-C-R
                       |   \
                       |    W
30                     |    G
                       |   /
       R-G-V-X-F-C-I
```

Kite form-5

```
35     R-G-G-R-L-X-Y-C-R
                       |   \
                       |    P
                       |    R
                       |   /
40     R-G-V-X-V-C-F
```

Bullet form-1

```
       R-G-G-R-L-C-Y-X-R
                       |   \
45                     |    R
                       |    R
                       |   /
       R-G-V-C-V-X-F
```

Bullet form-2

```
R-G-G-R-L-C-Y-X-R
                  \
                   R
                   R
                  /
R-G-V-C-I-X-F
```

Bullet form-3

```
R-G-G-G-L-C-Y-X-R
                  \
                   R
                   R
                  /
R-G-V-C-V-X-F
```

Bullet form-4

```
R-G-G-R-L-C-Y-X-R
                  \
                   W
                   G
                  /
R-G-V-C-F-X-I
```

Bullet form-5

```
R-G-G-R-L-C-Y-X-R
                  \
                   P
                   R
                  /
R-G-V-C-V-X-F
```

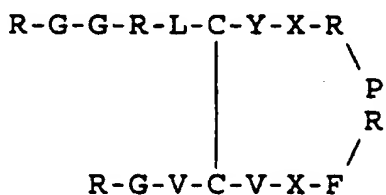**Snake form-1**:   R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

Snake form-2:   R-G-G-R-L-X-Y-X-R-R-R-F-X-I-X-V

Snake form-3:   R-G-G-G-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

Snake form-4:   R-G-G-R-X-L-X-Y-R-G-W-I-X-F-X-V-G-R

Snake form-5:   R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V-G-R

wherein X is as defined above.

Particularly preferred embodiments of X are those
wherein X is a small amino acid, especially S and A,
especially A.

Preparation of the Invention Compounds

The invention compounds, often designated herein
"protegrins" are essentially peptide backbones which may be
modified at the N- or C-terminus and also may contain one or

two cystine disulfide linkages. The peptides may first be
synthesized in noncyclized form. These peptides may then be
converted to the cyclic peptides if desired by standard
methods of cystine bond formation. As applied to the
5   protegrins herein, "cyclic forms" refers to those forms
which contain cyclic portions by virtue of the formation of
disulfide linkages between cysteine residues in the peptide.
If the straight-chain forms are preferred, it is preferable
to stabilize the sulfhydryl groups for any peptides of the
10 invention which contain two or more cysteine residues.

        Standard methods of synthesis of peptides the size of
protegrins are known. Most commonly used currently are
solid phase synthesis techniques; indeed, automated
equipment for systematically constructing peptide chains can
15 be purchased. Solution phase synthesis can also be used but
is considerably less convenient. When synthesized using
these standard techniques, amino acids not encoded by the
gene and D-enantiomers can be employed in the synthesis.
Thus, one very practical way to obtain the compounds of the
20 invention is to employ these standard chemical synthesis
techniques.

        In addition to providing the peptide backbone, the N-
and/or C-terminus can be derivatized, again using
conventional chemical techniques. The compounds of the
25 invention may optionally contain an acyl group, preferably
an acetyl group at the amino terminus. Methods for
acetylating or, more generally, acylating, the free amino
group at the N-terminus are generally known in the art; in
addition, the N-terminal amino acid may be supplied in the
30 synthesis in acylated form.

        At the carboxy terminus, the carboxyl group may, of
course, be present in the form of a salt; in the case of
pharmaceutical compositions this will be a pharmaceutically
acceptable salt. Suitable salts include those formed with
35 inorganic ions such as NH_4^+, Na^+, K^+, Mg^{++}, Ca^{++}, and the like
as well as salts formed with organic cations such as those
of caffeine and other highly substituted amines. The
carboxy terminus may also be esterified using alcohols of

the formula ROH wherein R is hydrocarbyl (1-6C) as defined
above. Similarly, the carboxy terminus may be amidated so
as to have the formula $-CONH_2$, $-CONHR$, or $-CONR_2$, wherein
each R is independently hydrocarbyl (1-6C) as herein
5   defined. Techniques for esterification and amidation as
well as neutralizing in the presence of base to form salts
are all standard organic chemical techniques.

If the peptides of the invention are prepared under
physiological conditions, the side-chain amino groups of the
10   basic amino acids will be in the form of the relevant acid
addition salts.

Formation of disulfide linkages, if desired, is
conducted in the presence of mild oxidizing agents.
Chemical oxidizing agents may be used, or the compounds may
15   simply be exposed to the oxygen of the air to effect these
linkages. Various methods are known in the art. Processes
useful for disulfide bond formation have been described by
Tam, J.P. et al., _Synthesis_ (1979) 955-957; Stewart, J.M. et
al, "Solid Phase Peptide Synthesis" 2d Ed. Pierce Chemical
20   Company Rockford, IL (1984); Ahmed A.K. et al., _J Biol Chem_
(1975) _250_:8477-8482 and Pennington M.W. et al., _Peptides_
_1990_, E. Giralt et al., ESCOM Leiden, The Netherlands (1991)
164-166. An additional alternative is described by Kamber,
B. et al., _Helv Chim Acta_ (1980) _63_:899-915. A method
25   conducted on solid supports is described by Albericio _Int J_
Pept Protein Res (1985) _26_:92-97.

A particularly preferred method is solution oxidation
using molecular oxygen. This method has been used by the
inventors herein to refold synthetic PG-1, PG-3 in its amide
30   or acid forms, _enantio_PG-1 and the two unisulfide PG-1
compounds (C_6-C_{15} and C_8-C_{13}). Recoveries are as high as
30%.

If the peptide backbone is comprised entirely of gene-
encoded amino acids, or if some portion of it is so
35   composed, the peptide or the relevant portion may also be
synthesized using recombinant DNA techniques. The DNA
encoding the peptides of the invention may itself be
synthesized using commercially available equipment; codon

choice can be integrated into the synthesis depending on the nature of the host. Alternatively, although less convenient, the DNA can be obtained, at least initially, by screening a cDNA library prepared from porcine leukocytes

5 using probes or PCR primers based on the sequences of the protegrins described herein. This results in recovery of the naturally occurring sequence encoding the protegrins of the invention. Obtention of this native sequence is significant for purposes other than the synthesis of the

10 protegrins per se; the availability of the naturally occurring sequences provides a useful probe to obtain corresponding DNA encoding protegrins of other species. Thus, cDNA libraries, for example, of leukocytes derived from other animals can be screened using the native DNA,

15 preferably under conditions of high stringency. High stringency is as defined by Maniatis, et al. Molecular Cloning: a Laboratory Manual 2nd Ed, Cold Spring Harbor Laboratory Press (1989), the relevant portions of which are incorporated herein by reference. This procedure also

20 permits recovery of allelic variants of these peptides from the same species.

Alternatively, the protegrins can be prepared by isolation from leukocytes of a desired species using techniques similar to those disclosed herein for the

25 isolation of porcine protegrins. In general, these techniques involve preparing a lysate of a leukocyte preparation, ultrafiltering the supernatant of the clarified lysate and recovering the ultrafiltrate. The ultrafiltrate is then subjected to chromatographic separation. The

30 location of fragments having antimicrobial and antiviral activity corresponding to protegrins can be assessed using criteria of molecular weight and assaying the fractions for the desired activities as described herein. The native forms of these peptides are believed to be the cyclic forms;

35 if desired, the linearalized forms can be prepared by treating the peptides with reducing agents and stabilizing the sulfhydryl groups that result.

Isolated and recombinantly produced forms of the protegrins may require subsequent derivatization to modify the N- and/or C-terminus and, depending on the isolation procedure, to effect the formation of cystine bonds as

5   described hereinabove. Depending on the host organism used for recombinant production and the animal source from which the protein is isolated, some or all of these conversions may already have been effected.

For recombinant production, the DNA encoding the

10   protegrins of the invention is included in an expression system which places these coding sequences under control of a suitable promoter and other control sequences compatible with an intended host cell. Types of host cells available span almost the entire range of the plant and animal

15   kingdoms. Thus, the protegrins of the invention could be produced in bacteria or yeast (to the extent that they can be produced in a nontoxic or refractile form or utilize resistant strains) as well as in animal cells, insect cells and plant cells. Indeed, modified plant cells can be used

20   to regenerate plants containing the relevant expression systems so that the resulting transgenic plant is capable of self protection vis-à-vis these infective agents.

The protegrins of the invention can be produced in a form that will result in their secretion from the host cell

25   by fusing to the DNA encoding the protegrin, a DNA encoding a suitable signal peptide, or may be produced intracellularly. They may also be produced as fusion proteins with additional amino acid sequence which may or may not need to be subsequently removed prior to the use of

30   these compounds as antimicrobials or antivirals.

Thus, the protegrins of the invention can be produced in a variety of modalities including chemical synthesis, recombinant production, isolation from natural sources, or some combination of these techniques.

35   Those members of the protegrin class which occur naturally are supplied in purified and isolated form. By "purified and isolated" is meant free from the environment in which the peptide normally occurs (in the case of such

naturally occurring peptides) and in a form where it can be used practically. Thus, "purified and isolated" form means that the peptide is substantially pure, i.e., more than 90% pure, preferably more than 95% pure and more preferably more
5   than 99% pure or is in a completely different context such as that of a pharmaceutical preparation.

Antibodies

Antibodies to the protegrins of the invention may also
10 be produced using standard immunological techniques for production of polyclonal antisera and, if desired, immortalizing the antibody-producing cells of the immunized host for sources of monoclonal antibody production. Techniques for producing antibodies to any substance of
15 interest are well known. It may be necessary to enhance the immunogenicity of the substance, particularly as here, where the material is only a short peptide, by coupling the hapten to a carrier. Suitable carriers for this purpose include substances which do not themselves produce an immune
20 response in the mammal to be administered the hapten-carrier conjugate. Common carriers used include keyhole limpet hemocyanin (KLH), diphtheria toxoid, serum albumin, and the viral coat protein of rotavirus, VP6. Coupling of the hapten to the carrier is effected by standard techniques
25 such as contacting the carrier with the peptide in the presence of a dehydrating agent such as dicyclohexylcarbodiimide or through the use of linkers such as those available through Pierce Chemical Company, Chicago, IL.
30 The protegrins of the invention in immunogenic form are then injected into a suitable mammalian host and antibody titers in the serum are monitored. It should be noted, however, that some forms of the protegrins require modification before they are able to raise antibodies, due
35 to their resistance to antigen processing. For example, the native form of PG-1, containing two cystine bridges is nonimmunogenic when administered without coupling to a larger carrier and was a poor immunogen even in the presence

of potent adjuvants and when coupled through glutaraldehyde
or to KLH. Applicants believe this to be due to its
resistance to attack by leukocyte serine proteases (human
PMN elastase and cathepsin G) as well as to attack by an
5   aspartic protease (pepsin) that resembles several macrophage
cathepsins. The lack of immunogenicity may therefore result
from resistance to processing to a linear form that can fit
in the antigen-presenting pocket of the presenting cell.
Immunogenecity of these forms of the protegrins can be
10   enhanced by cleaving the disulfide bonds.

        Polyclonal antisera may be harvested when titers are
sufficiently high. Alternatively, antibody-producing cells
of the host such as spleen cells or peripheral blood
lymphocytes may be harvested and immortalized. The
15   immortalized cells are then cloned as individual colonies
and screened for the production of the desired monoclonal
antibodies.

        The antibodies of the invention are, of course, useful
in immunoassays for determining the amount or presence of
20   the protegrins. Such assays are essential in quality
controlled production of compositions containing the
protegrins of the invention. In addition, the antibodies
can be used to assess the efficacy of recombinant production
of the protegrins, as well as screening expression libraries
25   for the presence of protegrin encoding genes.

Compositions Containing the Protegrins and Methods of Use

        The protegrins of the invention are effective in
inactivating a wide range of microbial and viral targets,
30   including gram-positive and gram-negative bacteria, yeast,
protozoa and certain strains of virus. Accordingly, they
can be used in disinfectant compositions and as
preservatives for materials such as foodstuffs, cosmetics,
medicaments, or other materials containing nutrients for
35   organisms. For use in such contexts, the protegrins are
supplied either as a single protegrin, in admixture with
several other protegrins, or in admixture with additional
antimicrobial agents. In general, as these are

preservatives in this context, they are usually present in
relatively low amounts, of less than 5%, by weight of the
total composition, more preferably less than 1%, still more
preferably less than 0.1%.

5   The peptides of the invention are also useful as
standards in antimicrobial assays and in assays for
determination of capability of test compounds to bind to
endotoxins such as lipopolysaccharides.

         For use as antimicrobials or antivirals for treatment
10   of animal subjects, the protegrins of the invention can be
formulated as pharmaceutical or veterinary compositions.
Depending on the subject to be treated, the mode of
administration, and the type of treatment desired -- e.g.,
prevention, prophylaxis, therapy; the protegrins are
15   formulated in ways consonant with these parameters. A
summary of such techniques is found in Remington's
Pharmaceutical Sciences, latest edition, Mack Publishing
Co., Easton, PA.

         The protegrins are particularly attractive as an active
20   ingredients pharmaceutical compositions useful in treatment
of sexually transmitted diseases, including those caused by
Chlamydia trachomatis, Treponema pallidum, Neisseria
gonorrhoeae, Trichomonas vaginalis, Herpes simplex type 2
and HIV. Topical formulations are preferred and include
25   creams, salves, oils, powders, gels and the like. Suitable
topical excipient are well known in the art and can be
adapted for particular uses by those of ordinary skill.

         In general, for use in treatment or prophylaxis of
STDs, the protegrins of the invention may be used alone or
30   in combination with other antibiotics such as erythromycin,
tetracycline, macrolides, for example azithromycin and the
cephalosporins. Depending on the mode of administration,
the protegrins will be formulated into suitable compositions
to permit facile delivery to the affected areas. The
35   protegrins may be used in forms containing one or two
disulfide bridges or may be in linear form. In addition,
use of the enantiomeric forms containing all D-amino acids
may confer advantages such as resistance to those proteases,

such as trypsin and chymotrypsin, to which the protegrins
containing L-amino acids are less resistant.

    The protegrins of the invention can be administered
singly or as mixtures of several protegrins or in
5   combination with other pharmaceutically active components.
The formulations may be prepared in a manner suitable for
systemic administration or topical or local administration.
Systemic formulations include those designed for injection
(e.g., intramuscular, intravenous or subcutaneous injection)
10 or may be prepared for transdermal, transmucosal, or oral
administration. The formulation will generally include a
diluent as well as, in some cases, adjuvants, buffers,
preservatives and the like. The protegrins can be
administered also in liposomal compositions or as
15 microemulsions.

    If administration is to be oral, the protegrins of the
invention must be protected from degradation in the stomach
using a suitable enteric coating. This may be avoided to
some extent by utilizing amino acids in the D-configuration,
20 thus providing resistance to protease. However, the peptide
is still susceptible to hydrolysis due to the acidic
conditions of the stomach; thus, some degree of enteric
coating may still be required.

    As described in the examples below, the peptides of the
25 invention retain their activity against microbes in the
context of borate solutions that are commonly used in eye
care products. It has also been shown that when tested for
antimicrobial activity against *E. coli* in the presence and
absence of lysozyme in borate buffered saline, that the
30 presence of lysozyme enhanced the effectiveness of PG-3.
This effect was more pronounced when the PG-3 was autoclaved
and similar patterns were obtained for both the free-acid
form and the amide. Accordingly, the protegrins may be used
as preservatives in such compositions or as antimicrobials
35 for treatment of eye infections.

    It is particularly important that the protegrins retain
their activity under physiological conditions including
relatively high saline and in the presence of serum. In

addition, the protegrins are not cytotoxic with respect to the cells of higher organisms. These properties, described herein below in the Examples, make them particularly suitable for *in vivo* and therapeutic use.

5   The protegrins of the invention may also be applied to plants or to their environment to prevent viral- and microbial-induced diseases in these plants. Suitable compositions for this use will typically contain a diluent as well as a spreading agent or other ancillary agreements

10   beneficial to the plant or to the environment.

Thus, the protegrins of the invention may be used in any context wherein an antimicrobial and/or antiviral action is required. This use may be an entirely *in vitro* use, or the peptides may be administered to organisms.

15   In addition, the antimicrobial or antiviral activity may be generated *in situ* by administering an expression system suitable for the production of the protegrins of the invention. Such expression systems can be supplied to plant and animal subjects using known techniques. For example, in

20   animals, pox-based expression vectors can be used to generate the peptides *in situ*. Similarly, plant cells can be transformed with expression vectors and then regenerated into whole plants which are capable of their own production of the peptides.

25   A particularly useful property of the protegrins is their activity in the presence of serum. Unlike defensins, protegrins are capable of exerting their antimicrobial effects in the presence of serum.

As shown hereinbelow, the protegrins are capable of

30   inactivating endotoxins derived from gram-negative bacteria -- i.e., lipopolysaccharides (LPS) -- in standard assays. Accordingly, the protegrins may be used under any circumstances where inactivation of LPS is desired. One such situation is in the treatment or amelioration of gram-

35   negative sepsis.

The protegrins of the invention, therefore, represent a peculiarly useful class of compounds because of the following properties:

1)   they have an antimicrobial effect with respect to a
broad spectrum of target microbial systems, including
viruses, including retroviruses, bacteria, fungi, yeast and
protozoa.

5   2)   Their antimicrobial activity is effective under
physiological conditions - i.e., physiological saline and in
the presence of serum.

3)   They are not toxic to the cells of higher
organisms.

10   4)   They can be prepared in nonimmunogenic form thus
extending the number of species to which they can be
administered.

5)   They can be prepared in forms which are resistant
to certain proteases suggesting they are antimicrobial even

15   in lysosomes.

6) They can be prepared in forms that resist
degradation when autoclaved, thus simplifying their
preparation as components of pharmaceuticals.

The following examples are intended to illustrate but

20   not to limit the invention.

Example 1
Isolation of PG-1, PG-2 and PG-3

Fresh porcine blood was collected into 15-liter vessels

25   containing 5% EDTA in normal saline, pH 7.4 as an
anticoagulant (33 ml/liter blood). The blood cells were
allowed to sediment for 90 minutes at room temperature and
the leukocyte-rich supernatant was removed and centrifuged
at 200 x g for 5.7 minutes. The pellets were pooled and

30   suspended in 0.84% ammonium chloride to lyse erythrocytes
and the resulting leukocytes (70-75% PMN, 5-10% eosinophils,
15-25% lymphocytes and monocytes) were washed in normal
saline, resuspended in ice-cold 10% acetic acid at 10^8/ml,
homogenized and stirred overnight at 4°C. The preparation

35   was centrifuged at 25,000 x g for 3 hours at 4°C and the
supernatant was lyophilized and weighed.

950 mg (dry weight) of lyophilized extract, which
contained 520 mg protein by BCA analysis, was stirred
overnight at 4°C in 100 ml of 10% acetic acid and then
centrifuged at 25,000 x g for 2 hours. The supernate was
5   removed and passed by pressure through a 50 ml stirred
ultracentrifugation cell (Amicon, Danvers, MA) that
contained a YM-5 filter. The ultrafiltrate (24.5 mg protein
by BCA) was concentrated to 3 ml by vacuum centrifugation
(SpeedVac Concentrator, Savant Instruments, Hicksville, NY),
10   applied to a 2.5 x 117 cm BioGel P10 column (Bio-Rad,
Hercules, CA) and eluted at 4°C with 5% acetic acid.

Fractions containing 6.6 ml were obtained. Fractions
were assayed by absorption at 280 nm and the elution pattern
is shown in Figure 1.

15   Aliquots (66 µl) of each fraction were dried by vacuum
centrifugation and resuspended in 6.6 µl of 0.01% acetic
acid. Five µl samples of this concentrate were tested for
antimicrobial activity against *E. coli* ML-35,
L. monocytogenes, strain EGD and *C. albicans*, strain 820,
20   using radiodiffusion and gel overlay techniques as described
by Lehrer, R.I. *et al.* J Immunol Meth (1991) 137:167-173.
Briefly, the underlay agars used for all organisms had a
final pH of 6.5 and contained 9 mM sodium phosphate/1 mM
sodium citrate buffer, 1% w/v agarose and 0.30 µg/ml
25   tryptocase soy broth powder (BBL Cockeysville, MD). The
units of activity in the radial diffusion assay were
measured as described; 10 units correspond to a 1 mm
diameter clear zone around the sample well. Activities
obtained for the various fractions are shown in Figure 2.
30   Activity was found in a large number of fractions.

The active fractions were further examined by acid-urea
PAGE (AU-PAGE) and SDS PAGE. Results of these analyses
showed that active antimicrobial peptides of the appropriate
molecular weight were present and concentrated in fractions
35   76-78.

Fractions 76-78 from the Biogel P10 column were then
pooled and chromatographed on a 1 x 25 cm Vydac 218 TP1010

column with a gradient (buffer A is 0.1% TFA; buffer B is
0.1% TFA in acetonitrile) the increase in acetonitrile
concentration was 1% per minute. The results, assessed in
terms of absorbance at 280 nm and at 225 nm are shown in

5   Figure 3. The peaks corresponding the three peptides
illustrated herein are labeled in the figure. The figure
also contains an inset which shows the results of an acid-
urea PAGE gel stained with Comassie Blue that contains a
starting mixture composed of the pooled fractions and the

10   individual PG species. These are labeled M, 1, 2 and 3 on
the inset. The results clearly show the presence of three
distinct proteins.

The isolated proteins were subjected to amino acid
analysis using three independent methods, and to Edman

15   degradation, chymotrypsin digestion, and fast atom
bombardment mass spectrometric analysis. The peptides,
named "protegrins", are shown to have the amino acid
sequences as follows:

    PG-1:   RGGRLCYCRRRFCVCVGR

20   PG-2:   RGGRLCYCRRRFCICV

    PG-3:   RGGGLCYCRRRFCVCVGR,
and are amidated at the C-terminus.

The amidation status of the isolated peptides was
established by synthesis of PG-3 both in the free carboxyl

25   and carboxyamidated forms. These synthetic peptides were
then compared to isolated PG-3 using AU-PAGE and also using
reverse-phase HPLC. In both cases, the native product
comigrated with the synthetic amidated form.

The location of the disulfide linkages in the isolated

30   protegrins was also studied using PG-2 as a model. The
determination was performed using sequential enzyme
digestion (chymotrypsin followed by thermolysin) with direct
analysis using LC-ESI-MS on the fragments obtained. The
results of these analyses showed that the two intramolecular

35   disulfide bonds were C_6-C_{15} and C_8-C_{13}. With the location of
the disulfides in these positions, the protegrin molecules

are likely to exist as anti-parallel β sheets similar to the tachyplesins in overall conformation.

The antimicrobial proteins above are present in much lower concentrations in initial extracts than are the rabbit
5 defensins in corresponding crude extracts where the defensins constitute more than 15% of the total protein in rabbit granulocytes. Using the AU-PAGE analytical method on the various stages of purification, the peptides are only faintly visible in the crude extracts, whereas corresponding
10 crude extracts of rabbit granulocytes clearly show the presence of the defensins. The peptides of the invention become clearly evident only after the ultrafiltration step.

Because the protegrins whose structures are set forth above show sequence homology to the decapeptide region
15 corresponding to residues 1-10 of rabbit defensin NP-3a in the decapeptide region at positions 4-13 of PG-3, the protegrins, and in particular PG-3, may share the property of defensin NP-3a in being capable of competitively antagonizing ACTH-mediated steroid synthesis by adrenocytes.
20 This property, called "corticostasis", may influence the effectiveness of the protegrins as antiinfectious agents when employed in vivo.

Example 2
Antimicrobial Activity
25   The radial diffusion assay in agarose gels described in Example 1 was also used to test the activity of the purified protegrins. Figures 4a, 4b and 4c show the results against three test organisms in units described as above. The
30 rabbit defensin (NP-1) and the human defensin (HNP-1) were used as controls.

Figure 4a shows that PG-1 and PG-3 are more effective against E. coli ML-35P than HNP-1 and only slightly less effective than NP-1. PG-1 and PH-3 were also effective
35 against Listeria monocytogenes, strain EGD as shown in Figure 4b. In Figure 4c, PG-1 and PG-3 were also shown effective against Candida albicans. In general, these

peptides are approximately as effective as rabbit defensin
NP-1 on a weight basis and are more effective than HNP-1.
In all cases, PG-2 was also effective against the three
organisms tested but was not as active as the other two
5   peptides.

In addition to its activity in inhibiting the growth of
the above-mentioned organisms, the PG-1 of the invention has
been shown directly to inhibit the growth of *Staphylococcus
aureus* (see Figure 4d) and *K. pneumoneae* 270 (Figure 4e).
10   HNP-1 used as a control was less effective against *S. aureus*
and almost entirely ineffective against *K. pneumoneae*.

The protegrins of the invention have also been tested
against various other organisms and show broad spectrum
activity. In addition to their effectiveness in inhibiting
15   the growth of or infection by microorganisms associated with
STDs as described in Example 9 hereinbelow, the protegrins
show strong activity against the following microorganisms in
addition to those tested hereinabove: *Pseudomonas
aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhimurium*,
20   *Staphylococcus aureus*, *Histoplasma capsulatum*, *Myobacterium
avium-intracellulare*, and *Mycobacterium tuberculosis*. The
protegrins showed only fair activity against *Vibrio
vulnificus* and were inactive against *Vibrio cholerae* and
Borrelia burgdorferi.
25

Example 3
Retention of Activity Under Certain Conditions
The antimicrobial activity of the invention compounds
was tested as set forth above, but under conditions of 100µM
30   NaCl and in the presence of 90% fetal calf serum. Figures
5a and 5b show that PG-1 and PG-3 retain their activity with
respect to *C. albicans* and *E. coli* respectively, even in the
presence of 100mM NaCl. Neither NP-1 nor HNP-1 have this
property. Figure 5c shows that although NP-1 and NHP-2 lose
35   their ability to inactivate *C. albicans* in 90% fetal calf
serum, inactivation by PG-3 is retained.

Accordingly, the protegrins of the invention retain
their antimicrobial properties under useful physiological
conditions, including isotonic and borate solutions
appropriate for use in eye care products.

5   In addition, synthetic PG-1 was tested with respect to
its activity against *E. coli* ML-35 (serum sensitive) in
underlayered gels containing only 10 mM sodium phosphate
buffer, pH 7.4 and a 1:100 dilution of trypticase soy broth,
both in the presence and absence of 2.5% normal human serum,
10   which is below the lytic concentration for this strain of *E.
coli*.   In the presence of serum, the minimal bacteriocidal
concentration was reduced from approximately 1.0 µg/ml to
about 0.1 µg/ml.   This type of effect was not observed
either for a linear fragment of cathepsin G or for the
15   defensin HNP-1.

Similarly, using *C. albicans* as a target organism,
underlayers were prepared with 10 mM sodium phosphate with
and without 10% normal human serum.   The minimal fungicidal
concentration fell from about 1.3 µg/ml in the absence of
20   serum to 0.14 µg/ml in its presence.   The serum itself at
this concentration did not effect *C. albicans*.

Thus, not only is the action of the protegrins not
inhibited by the presence of serum, it is enhanced thereby.
Similar results were obtained using *L. monocytogenes* as the
25   target organism.

The protegrins PG-1 and PG-3 were incubated for 4 hours
at pH 2.0 with 0.5 µg/ml pepsin and then neutralized.   The
residual antimicrobial activity against *C. albicans*, *E. coli*
and *L. monocytogenes* was assessed and found to be fully
30   retained.   Similar experiments show that these compounds are
not degraded by human leukocyte elastase or by human
leukocyte cathepsin G even when exposed to high
concentrations of these enzymes and at a pH of 7.0 - 8.0
favorable for proteolytic activity.   In addition, synthetic
35   PG-3 amide and synthetic PG-3 acid were autoclaved and
tested for antimicrobial activity against *E. coli*, *L.
monocyogenese* and *C. albicans*; retaining full antimicrobial

activity in all cases. It is possible that the stability of
these compounds to protease degradation and to autoclaving
is enhanced by the presence of disulfide bonds.

5   Example 4
                        Ability to Bind Endotoxin
        The protegrins of the invention were tested for their
ability to bind the lipid polysaccharide (LPS) of the gram-
negative bacterium *E. coli* strain 0.55B5. The assay was the
10 *Limulus* amebocyte lysate (LAL) test for endotoxins conducted
in the presence and absence of the test compounds. The test
was conducted using the procedure described in Sigma
Technical Bulletin No. 210 as revised in December 1992 and
published by Sigma Chemical Company, St. Louis, MO.
15   The LAL test is based on the ability of LPS to effect
gelation in the commercial reagent E-Toxate™ which is
prepared from the lysate of circulating amebocytes of the
Horseshoe Crab *Limulus polyphemus*. As described in the
technical bulletin, when exposed to minute quantities of
20 LPS, the lysate increases in opacity as well as viscosity
and may gel depending on the concentration of endotoxin.
The technical bulletin goes on to speculate that the
mechanism appears analogous to the clotting of mammalian
blood and involves the steps of activation of a trypsin-like
25 preclotting enzymes by the LPS in the presence of calcium
ion, followed by enzymic modifications of a "coagulogen" by
proteolysis to produce a clottable protein. These steps are
believed tied to the biologically active or "pyrogenic"
portion of the molecule. It has been shown previously that
30 detoxified LPS (or endotoxin) gives a negative LAL test.
        The test compounds were used at various concentrations
from 0.25 µg-10 µg in a final volume of 0.2 ml and the test
mixtures contained LPS at a final concentration of 0.05
endotoxin unit/ml and E-Toxate™ at the same concentration.
35 The test compounds were incubated together with the LPS for
15 minutes before the E-Toxate™ was added to a final volume

after E-Toxate™ addition of 0.2 ml.   The tubes were then
incubated for 30 minutes at 37°C and examined for the
formation of a gel.

5   Both isolated native protegrins (nPGs) and
synthetically prepared protegrins (sPGs) were tested.   The
sPGs were prepared with a carboxyl group at the C-terminus
or with an amidated C-terminus.   The nPGs are amidated at
the C-terminus.   Also tested were six different rabbit
defensins (NPs) and four native human defensins (HNPs).   The
10   results are shown in Table 1.

Table 1						
Peptide	10 µg	5 µg	2.5 µg	1.0 µg	0.5 µg	0.25 µg
nPG-1	no gel	no gel	no gel	no gel	+	+ +
nPG-2	no gel	no gel	no gel	no gel	+	+ +
nPG-3	no gel	no gel	trace	+ +	+ +	+ +
sPG-3 acid	no gel	no gel	trace	+ +	+ +	+ +
sPG-3 amide	no gel	no gel	no gel	+	+ +	+ +
NP-1	not tested	not tested	+ +	+ +	+ +	+ +
NP-2	trace	+	+	+ +	+ +	+ +
NP-3a	no gel	no gel	no gel	+ +	+ +	+ +
NP-3b	no gel	no gel	+	+ +	+ +	+ +
NP-4	not tested	not tested	+	+ +	+ +	+ +
NP-5	no gel	trace	+	+	+ +	+ +
HNP-1	no gel	+	+	+ +	+ +	+ +
HNP-2	trace	trace	trace	+	+	+ +
HNP-3	no gel	+	+	+ +	+ +	+ +
HNP-4	no gel	trace	trace	+ +	+	+ +

As seen from the results, all of the protegrins, both
synthetic and native, and both in the amidated and
15   nonamidated forms are able to bind sufficiently to LPS to
prevent any substantial gel formation at concentrations as
low as 2.5 µg/0.2 ml.   nPG-1 and nPG-2 are effective at
somewhat lower concentrations.   The protegrins were
substantially more effective than the NP or HNP test
20   compounds; the most effective among these controls was

- 40 -

NP-3a, a peptide whose primary sequence most closely
resembles that of the protegrins.

In a follow-up experiment, the concentration of LPS was
varied from 0.05-0.25 endotoxin units (E.U.) and synthetic
5   PG-3 amide was used as the test compound. The results are
shown in Table 2.

Table 2			
Endotoxin Units	0.25 E.U.	0.10 E.U.	0.05 E.U.
sPG-3 amide (2.5 µg)	no gel	no gel	no gel
sPG-3 amide (1.0 µg)	no gel	no gel	no gel
sPG-3 amide (0.5 µg)	+ +	+ +	no gel
no added protein	+ +	+ +	+ +

These results show that since inhibition of gelation
10 can be overcome by increasing the concentration of LPS,
interaction with LPS is responsible for the lack of
gelation, rather than interfering with the gelation enzyme
cascade.

15   Example 5
                  Activity of Linearalized Forms
nPG-1 and nPG-3 were converted to linear form using a
reducing agent to convert the disulfide linkages to
sulfhydryl groups, which were then stabilized by alkylating
20 with iodoacetamide.

The ability of both cyclic and linearalized PG-1 and
PG-3 to inhibit gelation in the standard LAL assay was
assessed then as described in Example 4 and the results are
shown in Table 3.

25

Table 3					
Peptide	5 µg	2.5 µg	1.0 µg	0.25 µg	
nPG-1	no gel	no gel	+ +	+ +	+ +
cam-nPG-1	no gel	no gel	+ +	+ +	+ +
nPG-3	no gel	no gel	+ +	+ +	+ +
cam-nPG-3	no gel	no gel	+ +	+ +	+ +

These results show that the linearalized and cyclic
forms of the protegrins are equally capable of inhibiting
gelation and binding to endotoxin.

The antimicrobial activity of the linearalized forms
5   was also compared with that of the native protegrins. Both
linearalized and cyclic forms of the protegrins tested
continue to show antimicrobial activity, although the
effectiveness of these peptides as antimicrobials depends on
the nature of the target organism and on the test
10   conditions. The antimicrobial activity of native PG-1 and
its linearalized form (cam-PG-1) and PG-3 and its
linearalized form (cam-PG-3) were tested according to the
procedure set forth in Example 1 as described by Lehrer,
R.I. et al. J Immunol Meth (1991) 137:167-173. The results
15   are set forth in Figures 6a-6f.

Figures 6a and 6b show the antimicrobial activity of
these peptides in the concentration range 20 µg/ml-125 µg/ml
with respect to E. coli ML-35P either in 10 mM phosphate-
citrate buffer, pH 6.5 (Figure 6a) or in the presence of
20   this buffer plus 100 mM NaCl (Figure 6b). Both protegrins
showed strong antimicrobial activity with respect to this
organism; the linear form was slightly more potent in the
presence of buffer alone than was the cyclic form; on the
other hand, the cyclic form was more potent than the linear
25   form under isotonic conditions.

Figures 6c and 6d show the antimicrobial effect with
respect to L. monocytogenes. In Figure 6c where the above-
mentioned buffer alone was used, both cyclic and
linearalized forms of the protegrins showed strong
30   antimicrobial activity and both were approximately equally
effective over the concentration range tested (20 µg/ml-125
µg/ml).

Figure 6d shows the effect with respect to
L. monocytogenes in the presence of this buffer plus 100 mM
35   NaCl over the same concentration range. The cyclic form
retained strong antimicrobial activity with a slightly
greater concentration dependence. Linearalization appeared

to lower the activity appreciably although high
concentrations were still able to show an antimicrobial
effect.

The yeast *C. albicans* was tested with the results shown
5   in Figures 6e and 6f. Figure 6e shows that all forms of
these protegrins were antimicrobial in a dose-dependent
manner over the above concentration range when tested in the
presence of 10 mM phosphate buffer alone, although the
linearalized peptides were very slightly less effective.
10   Figure 6f shows the results of the same assay run in the
presence of buffer plus 100 mM NaCl. While the cyclized
forms retained approximately the same level of antimicrobial
effect, the activity of the linearalized forms was greatly
diminished so that at concentrations below 100 μg/ml of the
15   protegrin, virtually no antimicrobial effect was seen.
However, at higher concentrations of 130 μg/ml, a moderate
antimicrobial effect was observed.

Thus, depending on the target microorganism and the
conditions used, both the cyclized and linearalized forms of
20   the protegrins have antimicrobial activity.

Example 6
Antimicrobial Activity Under Conditions
Suitable for Treatment of the Eye

25   Contact lens solutions are typically formulated with
borate buffered physiological saline and may or may not
contain EDTA in addition. Protegrins in the form of the
synthetic PG-3 amide and synthetic PG acid were tested
generally in the assay described in Example 1 wherein all
30   underlay gels contain 25 mM borate buffer, pH 7.4, 1% (v/v)
tryptocase soy broth (0.3 μg/ml TSB powder) and 1% agarose.
Additions included either 100 mM NaCl, 1 mM EDTA or a
combination thereof. Other test compounds used as controls
were the defensin NP-1 and lysozyme. Dose response curves
35   were determined.

Table 4 shows the estimated minimal bacteriocidal
concentrations in μg/ml of the various test compounds.

Table 4				
ESTIMATED MINIMAL FUNGICIDAL CONCENTRATIONS (µg/ml)				
Peptide	buffer	+ EDTA	+ NaCl	+ EDTA & NaCl
sPG-3 amide	13.0	9.5	4.1	3.1
sPG-3 acid	15.0	9.5	4.6	3.7
NP-1	35.0	45.0	>200	>200
lysozyme	75.0	45.0	>200	>200

Although protegrins are somewhat less active in 25 mM
borate buffered saline than in 25 mM phosphate buffer, the
5   antimicrobial activity is enhanced by adding physiological
saline and modestly enhanced by 1 mM EDTA, as shown in the
table.

A similar test was run with *Candida albicans* as the
target organism with the results shown in Table 5, which
10   also shows estimates of minimal fungicidal concentrations.

Table 5			
ESTIMATED MINIMAL FUNGICIDAL CONCENTRATIONS (µg/ml)			
Peptide	25 mM borate buffer	borate buffer + 120 mM NaCl	borate buffer + EDTA & NaCl
nPG-3	32.0	9.0	8.0
sPG-3 amide	19.0	7.7	7.0
sPG-3 acid	19.0	9.2	9.3
NP-1	23.0	60.0	65.0
HNP-1	25.0	>200	>200

Table 6 shows results of similar experiments conducted
with *L. monocytogenes* as the target.
15

Table 6			
ESTIMATED MINIMAL BACTERICIDAL CONCENTRATIONS (µg/ml)			
Peptide	25 mM borate buffer	borate buffer + 120 mM NaCl	borate buffer + EDTA & NaCl
nPG-3	25.0	7.0	5.7
sPG-3 amide	21.0	5.7	5.2
sPG-3 acid	30.0	7.0	7.0
NP-1	20.0	11.0	3.8
HNP-1	11.0	>200	>200

The results shown indicate that these compounds are capable of exerting their antimicrobial effects under conditions typically associated with conditions suitable for
5   eye care products.

Example 7
Recovery of cDNA Clones and of a
New Protegrin-Encoding cDNA
10   cDNA Generation and PCR Amplification.

Total RNA was extracted from the bone marrow cells of a young red Duroc pig with guanidinium thiocyanate. One µg of total RNA was used to synthesize the first strand cDNA, with 20 pmol Oligo(dT) primer and 200 U Moloney-murine leukemia
15 virus (M-MLV) reverse transcriptase (Clontech Laboratory, Palo Alto, CA) in a total reaction volume of 20 µl. Two PCR primers were prepared. The sense primer (5'-GTCGGAATTCATGGAGACCCAGAG (A or G) GCCAG-3') corresponded to the 5' regions of PG-2 and PR-39 cDNA and contained an
20 EcoRI restriction site. The antisense primer (5'-GTCGTCTAGA (C or G) GTTTCACAAGAATTTATTT-3') was complementary to 3' ends of PG-2 and PR-39 cDNA immediately preceding their poly A tails and contained an XbaI restriction site. PCR was carried out in a 50 µl volume using 1/10 volume of the
25 above pig cDNA as template, 25 pmol primers and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer-Cetus). The reaction was run for 30 cycles, with 1 min denaturation (94°C) and annealing (60°C) steps and a 2 min extension step (72°C) per cycle.
30   cDNA Cloning and Sequencing. The amplified cDNA was fractionated by preparative agarose electrophoresis and stained with ethidium bromide. The main fragment was cut out, digested with EcoR I and Xba I endonucleases (New England Biolabs, Beverly, MA), subcloned into a M13mp18
35 bacteriophage vector, and transformed into E. coli XL1-Blue MRF' competent cells (Stratagene, La Jolla, CA). DNA sequencing was performed with a kit (U.S. Biochemical Corp.,

Cleveland, OH). Nucleotide and protein sequences were
analyzed with PC-GENE (Intelligenetics, Palo Alto, CA).

Northern blots. Ten μg of total RNA was denatured in
50% formamide, separated by electrophoresis through 1%

5   agarose gels in 0.62 M formaldehyde, and blotted onto
GeneScreen Plus membranes (DuPont, Boston, MA) by capillary
transfer. The membrane was baked at 80°C for 2 h, and
hybridized with ^{32}P-labeled probe in rapid hybridization
buffer (Amersham, Arlington Height, IL).

10   The results of sequencing the various clones encoding
the various protegrins is summarized in Figure 7. The cDNA
sequences of protegrins PG-1, PG-3 and PG-4 contain 691
bases as had previously been shown for PG-2 by Storici, P.
et al. Biochem Biophys Res Comm (1993) 196:1363-1368. The

15 cDNAs show an upstream sequence encoding 110 amino acids
which appears identical for all protegrins. Additional
differences, which are quite slight in nature, are shown in
Figure 7.

The analysis showed the presence of the protegrin PG-4

20 having an amino acid sequence of Formula (1) wherein A_{10} is a
small amino acid and A_{11} is a hydrophobic amino acid as
distinguished from the previously known protegrins where
these residues are basic. The amino acid sequence of PG-4
is therefore RGGRLCYCRGWICFCVGRG, wherein 1, 2, or 3 amino

25 acids at the N-terminus may be deleted.

Additional clones were obtained by amplifying reverse
transcribed porcine bone cell RNA using an upstream primer
that corresponds to the 5' end of PG-2 and another cathelin-
associated peptide, PR39, (Agerbeth B et al., Eur J Biochem

30 (1991) 202:849-854; Storici, P et al., Biochem Biophys Res
Com (1993) 186:1058-1065) and downstream primer that matches
the region immediately preceding the poly A region. The
resulting approximately 0.7 kb PCR product was subcloned
into M13mp18 and recombinant plaques were chosen for

35 purification and sequencing. In this manner, the sequences
for the precursors of PG-1, PG-3 and PG-4 were recovered.
All of these peptides are encoded by a nucleotide sequence

which encodes a precursor containing additional amino acid
sequence upstream of A_1 of the compound of formula 1 (as
shown for PG-4 in Figure 7).

5   Example 8
          Recovery of Genomic DNA Encoding PG-1, PG-3, and PG-5
          High molecular genomic DNA was purified from pig white
blood cells with the QIAGEN blood DNA kit (QIAGEN,
Chatsworth, CA). To amplify protegrin (PG) genes, PCR as
10   performed using genomic DNA as a template.
          The sense primer (5'-GTCGGAATTCATGGAGACCCAGAG(A or
G)GCCAG-3') corresponded to the 5' regions of PG cDNAs, of
Example 7 and provided an EcoRI restriction site. The
antisense primer (5'-GTCGTCTAGA(C or
15   G)GTTTCACAAGAATTTATTT-3') was complementary to 3' ends of PG
cDNAs immediately preceding their poly(A) tails and provided
an XbaI restriction site. The reaction was carried out in a
total volume of 50 μl, which contained 200 ng of purified
pig genomic DNA, 25 pmoles of each primer, 1 μl of 10 mM
20   dNTP, 5 μl of 10X PCR buffer (200 mM Tris-HCl, 100 mM(NH$_4$)$_2$,
20 mM MgSO$_4$, 1% Triton X-100, 0.1% BSA), and 2.5 units of
cloned Pfu DNA polymerase (Stratagene, La Jolla, CA).
Thirty cycles were performed, each with 1 min of
denaturation at 94°C, 1 min of primer annealing at 55°C, 2
25   min of primer extension at 72°C, and a final extension step
at 72°C for 10 min.
          The amplified PCR product was digested with EcoRI and
XbaI, excised from the agarose gel, purified, and ligated
into pBluescript KS+ vector (Stratagene, La Jolla, CA) that
30   had been digested with EcoRI and XbaI and purified. Both
strands of DNA were sequenced by the dideoxy method using
the Sequenase version 2.0 kit (United States Biochemical,
Cleveland, OH), pBluescript universal primers and specific
oligomer primers based on PG genomic and cDNA sequences.
35   Computer analysis of the DNA sequences was performed using
the PC-Gene Program (Intelligenetics, Palo Alto, CA).

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A PCR product of about 1.85 kb was confirmed as
protegrin-related by hybridization with a protegrin-specific
oligonucleotide probe complementary to nucleotides 403-429
of the protegrin cDNA sequences. The PCR product was then
5   subcloned into pBluescript vector, and recombinant plasmids
were subjected to DNA purification and sequencing. Gene
sequences for three different protegrins were identified
PG-1, PG-3 and PG-5. The nucleotide sequences and deduced
amino acid sequences are shown in Figure 8.

10   Comparison of protegrin cDNAs and genes revealed that
the coding regions of protegrin genes consisted of four
exons, interrupted by three introns (Figures 8 and 9). The
first exon contained the 5' noncoding region and codons for
the first 66 amino acids of the protegrin prepropeptide,
15   including a 29 residue signal peptide and the first 37
cathelin residues. Exons II and III were relatively small,
only 108 and 72 bp respectively, and together contained the
next 60 cathelin residues. The final two cathelin residues
were on Exon IV, and were followed by the protegrin
20   sequences. The exon-intron splice site sequences are shown
in Table 7, and conform to the consensus rule: all introns
end on an AG doublet, preceded by a T/C rich stretch of 8-12
bases, while all introns start with GT, followed
predominantly by A/G A/G G sequence.

25

Table 7					
Exon-Intron Structure of the PG-1 Gene					
Exon	Size	5' splice donor	Intron	Size	3' splice acceptor
1	?+198	AAGGCCgtgagtcg	1	405	ttgaccagGACGAG
2	108	AACGGGgtgaggct	2	152	ccttccagCGGGTG
3	72	AATGAGgtgagtgg	3	596	ggtcacagGTTCAA
4	313				

The highly conserved cathelin region spans exons I-IV
and Exon IV contains the full sequence of the mature
protegrin peptide followed by an amidation consensus
30   sequence, a 3' untranslated region, and the putative
polyadenylation site. The three introns range in size from

152 to 596 bp. If the protegrin genes are representative of
other cathelin-like genes, the third intron of cathelin-
associated peptides will be found to separate all but the
last two residues of the highly conserved cathelin region
5   from the variable antimicrobial peptides encoded in Exon IV.
Such a layout would favor recombination mechanisms involving
association of diverse Exon IVs with the first three exons
specifying cathelin containing prepro-regions.

The family of naturally occurring protegrins thus
10   contains at least 5 members. Figure 10 shows a comparison
of the amino acid sequences of the five protegrins found so
far in porcine leukocytes. There is complete homology in
positions 1-3, 5-9, 13 and 15-16.

Homology search of protegrin genes against the
15   EMBL/GenBank identified no significantly homologous genes.
More specifically, the gene structures and nucleotide
sequences of protegrins were very different from those of
defensins, which contain three exons in myeloid defensin
genes, and two exons in enteric defensin genes. As
20   expected, the search yielded the large family of cDNAs
corresponding to cathelin-associated bovine, porcine and
rabbit leukocyte peptides.

To assess protegrin-related genes further, we screened
a porcine genomic library of approximately 2.3 x 10^5 clones
25   in EMBL-3 SP6/T7 with the ^{32}P-labeled protegrin cDNA, and
identified 45 hybridizing clones.

A porcine liver genomic library in EMBL3 SP6/T7 phages
was purchased from Clontech (Palo Alto, CA). *E. coli* strain
K803 was used as a host, and DNA from phage plaques was
30   transferred onto nylon membranes (DuPont, Boston, MA). The
filters were hybridized with ^{32}P-labeled porcine 691 PG-3
cDNA. The filters were washed several times, finally at 60°C
in 0.1x SSC and 0.1% SDS, and exposed to x-ray film with an
intensifying screen at -70°C. Positive clones were subjected
35   to two additional rounds of plaque purification at low
density.

DNA purified from hybridizing clones was digested with various restriction endonucleases (New England Biolabs, Beverly, MA), fractionated on 0.8% agarose gels, and transferred onto GeneScreen Plus membrane (DuPont, Boston,

5   MA). The hybridization probes were labeled with ^{32}P and included porcine PG-3 cDNA, and 5'-labeled protegrin-specific oligonucleotide complementary to nt 403-429 of PG-1, 2 and 3 cDNAs. For the cDNA probe, the hybridization and washing conditions were carried out as for the library

10 screening. For the oligonucleotide probe, the membranes were washed at 42°C in 0.1x SSC, 0.1% SDS.

Southern blot analysis was carried out with purified DNA from positive clones by hybridization with protegrin cDNA and a protegrin specific oligonucleotide complementary

15 to nt 403-429 of protegrin cDNA sequences. Although all of the clones hybridized with the complete cDNA probe, only about half of them hybridized with the protegrin-specific probe. A specific oligonucleotide probe for porcine prophenin, another cathelin-associated porcine leukocyte-

20 derived antimicrobial peptide, hybridized to several of the nonprotegrin clones. These results confirm a) that the conserved proregion homologous to cathelin is present within the same gene as the mature antimicrobial peptides and is not added on by posttranscriptional events, and b) that the

25 protegrins account for about half of the cathelin-related genes in the pig.

A synthetic peptide corresponding to the amino acid sequence of PG-5 was prepared and tested with respect to antimicrobial activity against *E. coli*, *L. monocytogenes* and

30 *C. albicans*. The results were compared to those obtained with a synthetically prepared PG-1. The results are shown in Figures 11a-11c. As shown in these graphical representations of the results, PG-5 has comparable antimicrobial activity to PG-1 against all three organisms

35 tested.

Example 9

Preparation of *EnantioPG-1*

Using standard solid phase techniques, a protegrin having the amino acid sequence of PG-1, but wherein every amino acid is in the D form was prepared. This form of

5   protegrin was tested against *E. coli*, *L. monocytogenes*, *C. albicans* and other microbes in the absence and presence of protease and otherwise as described for the radiodiffusion assay in agarose gels set forth in Example 1. The results are shown in Figures 12a-12g.

10   Figure 12a shows that both native PG-1 and *enantioPG-1* in the absence of protease are equally effective in inhibiting the growth of *E. coli*. Figure 12b shows that neither trypsin nor chymotrypsin inhibits the antibacterial effect of *enantioPG-1*. Figure 12c shows that in the

15   presence of these proteolytic enzymes, the ability of native PG-1 to inhibit the growth of *L. monocytogenes* is adversely affected, although, as shown in Figure 12d, in the absence of these proteases PG-1 is comparably active to an *enantioPG-1*.

20

Example 10
Activity of the Protegrins Against STD Pathogens

Table 8 summarizes the activity of the protegrin PG-1 as compared to the defensin HNP-1 against growth of STD

25   pathogens. In these results, "active" means that the peptide was effective at less than 10 μg/ml; moderately active indicates that it was active at 10-25 μg/ml; and slightly active means activity at 25-50 μg/ml. If no effect was obtained at 50-200 μg/ml the compound was considered

30   inactive.

Table 8		
Activity against human STD pathogens	Protegrin PG-1	Defensin HNP-1
HIV-1	Active	Slightly active
Chlamydia trachomatis	Active	Slightly active
Treponema pallidum	Active	Inactive
Neisseria gonorrhoeae	Active	Inactive
Trichomonas vaginalis	Moderately active	Inactive
Herpes simplex type 2	Moderately active	Slightly active
Herpes simplex type 1	Inactive	Slightly active
Hemophilus ducreyi	Not tested	Not tested
Human papilloma virus	Not tested	Not tested

Chlamydia trachomatis

Unlike other bacteria associated with STDs, *Chlamydia*
requires an intracellular habitat for metabolic activity and
5   binary fission. The life cycle is as follows: there is an
extracellular form which is a metabolically inactive
particle somewhat sporelike in its behavior, referred to as
an elementary body (EB). The EB attaches to the host cell
and is ingested to form an internal vacuolar space often ⇀
10   called an "inclusion". The bacterium reorganizes to the
delicate reticulate body (RB) which is noninfective but
metabolically active and which over a 48-72 hour period
undergoes reformation to the EB state. The EBs are then
released from the cell. Rather than a peptidoglycan layer,
15   *Chlamydia* contains multiple disulfide linkages in cysteine-
rich proteins for protection in the EB stage.

The protegrins of the invention were tested for their
antimicrobial activity against *Chlamydia* using the "gold
standard" chlamydial culture system for clinical specimens
20   described by Clarke, L.M. in <u>Clinical Microbiology
Procedures Handbook II</u> (1992), Isenberg, H.T. Ed. Am. Soc.
Microbiol. Washington, D.C.; pp. 8.0.1 to 8.24.3.9.
Briefly, McCoy cells (a mouse cell line) in cycloheximide

EMEM with 10% fetal bovine serum (FBS) are used as hosts.
Prior to chlamydial inoculation, the maintenance medium is
aspirated without disruption of the cell layer and the cell
layer is maintained on a cover slip in a standard vial.

5   Each vial is then inoculated with 100-300 μL inoculum and
centrifuged at 3500 x g for one hour at 20°C. The fluid is
then aspirated and 1 ml of EMEM is added. The vials are
capped and incubated at 37°C for 48 hours. After 48 hours
the medium is again aspirated, coverslips are rinsed twice

10   with PBS and fixed with 300 μL EtOH for 10 minutes. The
EtOH is aspirated and the vials are allowed to dry; then one
drop PBS plus 30 μL Syva Microtrak monoclonal antibody to
the major outer membrane protein of *Chlamydia* is added for
staining. After 37°C incubation for 30 minutes, the cells

15   are washed with distilled water and examined for inclusions
which are easily recognizable as bright, apple-green-
staining cytoplasmic vacuoles. They represent the
equivalent of a colony of free-living bacteria on standard
bacterial culture media.

20   In the assays conducted below, *C. trachomatis* serovar
L2 (L2/434Bu) described by Kuo, C.C. *et al.* in <u>Nongynococcal
Urethritis and Related Infections</u> (1977), Taylor-Robinson,
D. *et al.* Ed. Am. Soc. Microbiol. Washington, D.C., pp. 322-
326 was used. The seed is prepared from a sonicated culture

25   in L929 mouse fibroblast cells, and partially purified by
centrifugation. Since host protein is still present in the
seed aliquots, each seed batch is titered at the time of
preparation with serial ten-fold dilutions to 2×10^{-9}. The
seed containing 9.2×10^{6} IFU/ml is thawed quickly at 37°C

30   and diluted to 10^{-2} with sucrose/phosphate salts/glycine to
produce IFU of about 200 after room temperature
preincubation and to dilute background eukaryotic protein.

             In the initial assays, the peptides to be tested were
prepared as stock solutions in 0.01% glacial acetic acid.

35   100 μL of the diluted chlamydial seed was aliquoted into 1.5
ml eppendorf tubes and 200 μL of the antibiotic peptide was

added per tube. Aliquots of the peptide stock (and
controls) were incubated with the seed at room temperature
for one hour, two hours and four hours. About 10 minutes
before the end of each incubation period, maintenance media
5   were aspirated from the McCoy vials in preparation for
standard inoculation and culture. Culture was then
performed in the presence and absence of the peptides; in
some cases, the peptides were added to final concentration
in the culture media in addition to the preculture
10   incubation. The test was evaluated microscopically.

The results using 50 µg of protegrin per addition were
dramatic. In control cultures, where no peptides were
added, 222-460 inclusions were counted. In all protocols
where protegrin was added either before the *Chlamydia* seed
15   was added to the cells or both before and after, no
inclusions were found. Similar results were obtained with
20 µg additions of tachyplesin. The defensins NP-1 and
HNP-1 had lesser protective effects. In summary, the
protegrins tested show antimicrobial against *Chlamydia*.
20   In the next series of experiments, various
concentrations of protegrin (1 µg, 12.5 µg, 25 µg and 50 µg)
were used in the two-hour preincubation. Concentrations as
low as 12.5 µg lowered the number of inclusions to zero.
Even at a concentration of 1 µg/ml, the number of inclusions
25   was lowered dramatically from about 110 to about 30.

In the next set of experiments, the effect of the
presence of serum was tested. The *Chlamydia* seed was
preincubated for two hours with and without 10% FBS and also
with or without protegrin at 25 µg. Protegrin was highly
30   effective both with and without serum, whereas human
defensin HNP-2, used as a control, was reasonably effective
in the absence of serum but only marginally effective in its
presence.

The experiments were repeated but adding 25 µg of
35   protegrin one after the start of the chlamydial culture,
i.e., after centrifugation and final medium mix and one hour
into the beginning of the 48-hour culture period. Protegrin

reduced the number of inclusions by approximately 57% from
untreated controls although HNP-2 was completely
ineffective. Finally, the protegrin (at 25 μg) was added to
the chlamydial seed and the mix then immediately cultured.
5   In this case, without preincubation and without the one-hour
post-infection gap, protegrin was minimally effective
without or without serum.

The effect of serum is particularly important since for
a topical agent to be effective in combatting *Chlamydia*
10   infection, it must act in the presence of serum.

In addition, there are several mouse-based models for
Chlamydia infection which can be used to assess the efficacy
of the protegrins. These include those described by Patton,
D.L. *et al.* in Chlamydial Infections (1990) Bowie, W.R. *et*
15   *al.* Eds. Cambridge University Press NY pp. 223-231; Swenson,
C.E. *et al.* J. Infect. Dis. (1983) pp. 1101-1107, and
Barron, A.L. *et al.* J. Infect. Dis. (1981) 143:63-66.

Neisseria gonorrhoeae

20   In more detail, the ability of the protegrins to
inhibit *N. gonorrhoeae* was tested by a modification of the
method of Miyasaki et al., Antimicrob Agent Chemother (1993)
37:2710-2715. Nonpiliated transparent variants of strains
FA 19 and F 62 were propagated on GCB agar plates containing
25   glucose and iron supplements overnight at 37°C under 3.8%
V/V CO_2. These strains were chosen for their adaptability
to the assay.

The overnight growth is removed from the agar plate and
suspended in GCB broth containing supplements and sodium
30   bicarbonate and grown with shaking at 37°C to mid log phase.
The culture is diluted 1:100 in GCB broth to give about 10^6
CFU/ml and serial dilutions were plated onto GCB agar.

The peptides are dissolved in 0.01% v/v acetic acid to
give a 1 mg/ml stock solution and serially diluted. Ten μl
35   of each dilution is added to a sterile polystyrene tube
containing 90 μl of diluted bacteria and the tubes are
shaken at 37°C for 45 minutes. The contents are serially

diluted 1:10 and plated on to GCB agar plates which are incubated in a CO_2 incubator. CFU are counted after 24 hours and the log bactericidal activity calculated.

5   Native PG-1, synthetic PG-1, synthetic PG-3 amide and synthetic PG-3 without amidation all gave over a 5 log reduction in CFU per ml in this assay. Native PG-2 (containing 16 amino acids) gave a 2.6 fold reduction.

In addition enantioPG-1, the unidisulfide PG-1 (C_6-C_{15}), and unisulfide PG-1 (C_8-C_{13}) gave over a 5-fold log reduction
10   in CFU/ml in this assay.

Treponema pallidum

Bactериоcidal activity against this organism, which is the etiologic agent of syphilis, was also tested. Peptides
15   were evaluated at a series of concentrations of 1.758 μg to 56.25 μg in 90 μl of unheated normal rabbit serum. The serum served as a nutrient for the spirochetes to allow their survival during incubation as well as providing a source of complement. Ten μl of a suspension of T. pallidum
20   containing about 5 x 10^7/μl organisms was added to each tube and the mixtures with the appropriate peptides were incubated at 34°C under 95% N_2 and 5% CO_2. At time zero, just prior to incubation, 4 hours and 16 hours, 25 randomly selected organisms were examined for the presence or absence
25   of motility. The 50% immobilizing end point (IE_{50}) was calculated to indicate the concentration needed to immobilize 50% of the spirochetes. In the presence of PG-1, the IE_{50} at 0 and 4 hours was 2.717 μg and < 1.758 μg, respectively. Tachyplesin IE_{50}'s were 5.231 μg and 2.539 μg
30   for 0 and 4 hours. This was in contrast to HNP and NP preparations which showed little immobilizing ability.

Herpes Simplex Virus

Using viral stocks prepared in VERO cells, grown in
35   minimal essential medium (MEM) with 2% fetal calf serum, the effect of various peptides on HSV 1 MacIntyre strain, a pool of ten clinical HSV 1 isolates, HSV-2G, and a pool of ten

clinical HSV 2 isolates, all sensitive to 3 μM acyclovir
were tested. Two fibroblast cell lines, human W138 and
equine CCL57, were used as targets and tests were done by
direct viral neutralization and delayed peptide addition.

5   In the direct neutralization format, the virus was
preincubated with the peptides for 90 min before it was
added to the tissue culture monolayers. In the delayed
peptide addition format, the virus was added and allowed 50
min to adsorb to the target cells, then the monolayers were
10 washed and peptides were added for 90 min. Finally, the
monolayer was washed to remove the peptide and the cells
were fed with peptide-free MEM and cultured until the
untreated infected monolayers exhibited 4+ cytopathic effect
(CPE) (about 60 hours).

15   Antiviral activity was seen in both formats, but was
more pronounced with the delayed peptide addition mode. In
experiments performed with W138 and CCL57 cells in the
direct neutralization format, PG-1 completely prevented HSV-
2G from causing CPE at concentrations of 50 μg/ml and 25
20 μg/ml, but these concentrations afforded no protection
against HSV-1, which produced 4+ CPE.

        In the delayed peptide addition format, PG-1 completely
prevented CPE by HSV-2G at 35 μg/ml and 50 μg/ml and it also
fully protected against the clinical HSV-2 pool at both
25 concentrations.

        Thus, PG-1 protected human and animal cells from
infection by laboratory and clinical strains of HSV-2, even
when the peptides were added as late as 60 min after the
virus had been introduced into the cell culture.

30

Trichomonas vaginallis

        Trichomonas vaginallis strain C1 (ATCC 30001) was grown
as described by Gorrell, T.E. et al, Carlsberg Res Comm
(1984) 49:259-268. In experiments performed in RPMI + 1%
35 heat-activated fetal calf serum, within a few minutes after
exposure to 50 μg/ml PG-1, _T. vaginallis_ (heretofore
vigorously motile) became stationary. Soon thereafter, the

organisms became permeable to trypan blue, and, over the
ensuing 15-30 minutes, lysed. As expected, such organisms
failed to grow when introduced into their customary growth
medium (Diamond's medium). Organisms exposed to 25 µg/ml of
5   PG-3 retained their motility.

Initial studies with two highly metronidazole-resistant
clinical isolates of *T. vaginallis*, strains MR and TV showed
both were susceptible to PG-1, including the C_8-C_{13} and C_6-
C_{15} uni-disulfides and *enantio*PG-1 at concentrations of 100
10 and 50 µg/ml.

Example 11
Antiretroviral Activity

Both synthetic and native PG-1 and native PG-2 were
15 tested for antiviral activity against strains of HIV using
the method described in Miles, S.A. et al., Blood (1991)
78:3200-3208. Briefly, the mononuclear cell fraction is
recovered from normal donor leukopacs from the American Red
Cross using a Ficoll-hypaque density gradient. The
20 mononuclear cells are resuspended at 1 x 10^6 cells per ml in
RPMI 1640 medium with 20% fetal bovine serum, 1% penn/strep
with fungizone and 0.5% PHA and incubated 24 hours at 37°C
in 5% CO_2. The cells are centrifuged, washed and then
expanded for 24 hours in growth medium.

25 Non-laboratory adapted, cloned HIV_{JR-CSF} and HIV_{JR-FL}
were electroporated into the human peripheral blood
mononuclear cells prepared as described above. Titers were
determined and in general, multiplicities of infection (MOI)
of about 4,000 infectious units per cell are used (which
30 corresponds to 25-40 picograms per ml HIV p24 antigen in the
supernatant).

In the assay, the HIV stocks prepared as above were
diluted to the correct MOI and the PBM are added to 24 well
plates at a concentration of 2 x 10^6 per ml. One µl total
35 volume is added to each well. The peptide to be tested is
added in growth medium to achieve the final desired
concentration. Then the appropriate number of MOI are

added. To assay viral growth, 200 µl of supernatant is
removed on days 3 and 7 and the concentration of p24 antigen
is determined using a commercial assay (Coulter Immunology,
Hialeah, Florida). Controls include duplicate wells
5   containing cells alone, cells plus peptide at 5 µg/ml cells
with virus but not peptide and cells with virus in the
presence of AZT at 10^{-5} M - 10^{-8} M.

Using this assay, it was demonstrated that both natural
and synthetic PG-1 completely inhibit HIV infection at
10   concentrations between 1-5 µg/ml; IC_{90} was < 5 µg/ml. The
time of addition of peptide was then varied. Cells
pretreated for 2 hours prior to addition of virus, at the
time of addition of virus, or 2 hours after infection showed
antiviral activity for the peptide. However, if PG-1 was
15   added 24 hours after infection, there was no antiviral
activity.

Further, PG-2 shows similar activity but at a level
approximately 5-fold less. Alternative antibiotics such as
human defensins and rabbit defensins lacked potent activity
20   in this assay. The results were similar for both HIV_{JR-CSF}
and HIV_{JR-FL} which are non-laboratory adapted isolates
(Koyanagi, Y.S. et al, Science (1987) 236:819-822).

The protegrins show similar activity with respect to
other retroviruses.
25

Example 12
Preparation of Modified Protegrins: Kite and Bullet Forms
The kite and bullet forms of PG-1 wherein all X are
alanine were synthesized using conventional Fmoc chemistry.
30   The crude synthetic peptide was reduced by adding
dithiothreitol (DTT) equal in weight to the synthetic
peptide which had been dissolved at 10 mg peptide/ml in a
solution containing 6 molar guanidine HCl, 0.5 molar tris
buffer, and 2 mM EDTA, pH 8.05 and incubated for two hours
35   at 52°C under nitrogen. The mixture was passed through a
0.45 µ filter, acidified with 1/20 (v/v) glacial acidic acid
and subjected to conventional RP-HPLC purification with a

C-18 column. HPLC-purified, reduced synthetic bullet and
kite PG-1 were partially concentrated by vacuum
centrifugation in a speed vac and allowed to fold for 24
hours at room temperature in ambient air in 0.1 M Tris
5   pH 7.7 at low concentration (0.1 mg peptide/ml) to minimize
formation of interchain cystine disulfides. The mixture was
then concentrated and acidified with HOAC to a final
concentration of 5% and subjected to RP-HPLC purification.
         The purity of the final products bullet and kite PG-1
10   was verified by AU-PAGE, analytical HPLC, and FAB-mass spec.
AU-PAGE showed a single band for the final product in each
case. The observed MH+ mass values were 2093 in both cases.

Example 13
15   ## Antimicrobial Activity of the Kite and Bullet Forms
         The kite and bullet PG-1 compounds prepared in Example
12 were tested for antimicrobial activity using the radial
diffusion assay described in Example 1 as published by
Lehrer, R.I. et al., *J Immunol Meth* (1991) 137:167-173,
20   except that the underlay agars contained 10 mm sodium
phosphate buffer with a final pH of 7.4. As described in
Example 1, 0.3 mg/ml tripticase soy broth powder and 1%
agarose were used as well in the underlay agar. In some
cases 100 mM NaCl or RPMI plus 2.5% normal human serum (NHS)
25   was added to the agar.
         In a first set of determinations, the bullet and kite
forms of PG-1 were tested for antimicrobial activity against
L. monocytogenes, *E. faecium* (VR) or *S. aureus* under these
three sets of conditions. Figure 13 shows the result.
30   As shown, the bullet and kite forms were roughly
equally effective against these three bacteria using
standard assay conditions. When 100 mM NaCl was added to
the agar, however, the kite forms appeared slightly less
active than the bullet forms which appear to have slightly
35   enhanced antimicrobial activity against all three stains
except *S. aureus* under these conditions. Similarly, when
RPMI plus 2.5% NHS were added, the bullet forms were again
more effective than the kite forms. The activity of the kit

form versus *E. faecium* was significantly less under these
conditions.

As shown in Figure 14, these forms of PG-1 were also
tested against *E. coli*, *K. pneumoniae* and *P. aeruginosa*.
5 All three microorganisms were inhibited by both kite and
bullet forms under standard conditions. This antimicrobial
activity was maintained also at 100 mM NaCl and RPMI plus
NHS.

10   Example 14
                 Synthesis of the Snake Form of PG-1
The snake form of PG-1 wherein all X are alanine was
performed using standard methods by Synpep Inc., Dublin, CA
and the MH+ value in FAB-mass spec was 2031.3 as expected.
15 The snake form was purified to homogeneity by RP-HPLC.

                              Example 15
               Antimicrobial Activity of Snake PG-1
Snake PG-1 was tested with respect to the same six
20 organisms and using the same conditions as set forth in
Example 13 with respect to the bullet and kite forms of
PG-1. The results are shown in Figures 15 and 16. In this
case, the native two-cystine form of PG-1 (native) was used
as a control. While the snake form shows somewhat superior
25 activity with respect to *L. monocytogenes*, *E. faecium*, and
S. aureus under standard conditions, it is notably less
effective than the native form in the presence of either
100 mM NaCl or RPMI plus NHS. The same pattern is followed,
as shown in Figure 16 when the test organisms are *E. coli*,
30 *K. pneumoniae*, and *P. aeruginosa*.

                              Example 16
           Minimal Inhibitory Concentrations of Protegrins
The minimal inhibitory concentrations (MICs) of a
35 variety of protegrins were determined against the following
organisms: methicillin resistant *Staphylococcus aureus*

(MRSA), *Pseudomonas aeruginosa* (Psa), vancomycin resistant *Enterococcus fecium* (VREF), *Candida albicans* (Candid) and *Escherichia coli* (E. Co), and are shown in Table 9.

Table 9 Peptides with 17-18 Amino Acids						
	SEQUENCE	MRSA	Psa	VREF	Candid	E. Co
IB-247	RGGRLCYCRRRFCVCVGR-OH	1.5	0.11		1.2	0.6
IB-249	RGGGLCYCRRRFCVCVGR-OH				3.29	0.4
IB-223	RGGGLCYCRRGFCVCFGR	1.93	0.14		1.62	
IB-224	RGGGLCYCRRPFCVCVGR	3.1	0.06		7.69	0.15
IB-324	RGGGLCYCRPRFCVCVGR-OH				17.7	3.51
IB-341	RGGRLCYCRXRFCVCVGR-OH (X=NMeG)	5.33	2	1		
IB-342	RGGRLCYCRXRFCVCVGR (X=NMeG)	4	1.67	0.83		
IB-384	RGGRLCYCXGRFCVCVGR (X=Cit)					
IB-398	RGGRVCYCRGRFCVCVGR	8	1			
IB-399	RGGRVCYCRGRFCVCVGR-OH					
IB-218	RGGGLCYCFPKFCVCVGR	3.48	1.2		15.96	
IB-349	RGGRLCYCRXR-Cha-CVCWGR (X=NMeG)					
IB-350	RGGRWCVCRXR-Cha-CYCVGR (X=NMeG)					
IB-394	RGGRWCVCRGR-cha-CYCVGR					
IB-416	RGGRLCYCRRRFC-NMeV-CVGR					
IB-400	RGGRVCYCRGRFCVCV	8	2			
IB-401	RGGRVCYCRGRFCVCV-OH	64	1			
	Uni-Disulfide Protegrins					
IB-214	RGGGLCYARGWIAFCVGR	2.1	0.59		32.6	0.81
IB-216	RGGGLCYARGFIAVCFGR	19	14		65.8	3.27
IB-225	RGGGLCYARPRFAVCVGR					
IB-226	RGGGLCYTRPRFTVCVGR	8.7	0.07			1.53
IB-227	RGGGLCYARKGFAVCVGR	>128	0.01			2.65
IB-288	RGGRLCYARRRFAVCVGR-OH		0.05		1.6	0.4
IB-289	RGGRLCYARRRFAVCVGR		0.05		1.6	0.4

5

Claims

    1.   A purified and isolated or recombinantly produced compound of the formula

5

$$A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-(A_{17}-A_{18}) \qquad (1)$$

    and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof, which is either in the

10 optionally -SH stabilized linear or in a cystine-bridged form

    wherein A_1 is a basic amino acid;

    each of A_2 and A_3 is independently a small amino acid;

    each of A_5, A_7, A_{14} is independently a hydrophobic amino

15 acid;

    A_4 is a basic or a small amino acid;

    each of A_9, A_{12} and A_{16} is independently a basic, a hydrophobic, a neutral/polar or a small amino acid;

    each of A_{10} and A_{11} is independently a basic, a

20 neutral/polar, a hydrophobic or a small amino acid or is proline;

    A_{17} is not present or, if present, is a basic, a neutral/polar, a hydrophobic or a small amino acid;

    A_{18} is not present or, if present, is a basic, a

25 hydrophobic, a neutral/polar or a small amino acid, or a

    modified form of Formula (1) and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof wherein at least one of the 4 cysteines is independently replaced by a hydrophobic amino acid or a

30 small amino acid;

    with the proviso that the compound of Formula (1) must have a charge of +3 or greater.

    2.   The compound of claim 1 which contains two cystine

35 bridges.

3.   The compound of claim 1 which contains one cystine
bridge, which is C_6-C_{15} or C_8-C_{13}.

4.   The compound of claim 1 which is in the linear
5   form.

5.   The compound of any of claims 1-4 wherein the
C-terminal carboxyl is of the formula selected from the
group consisting of COOH or the salts thereof; COOR, $CONH_2$,
10   CONHR, and $CONR_2$ wherein each R is independently
hydrocarbyl(1-6C);
        and/or wherein the amino group at the N-terminus is of
the formula NH_2 or NHCOR wherein R is hydrocarbyl(1-6C);
        and/or wherein each of A_1 and A_9 is independently
15   selected from the group consisting of R, K and Har;
        and/or wherein each of A_2 and A_3 is selected
independently from the group consisting of G, A, S and T;
        and/or wherein A_4 is R or G;
        and/or wherein each of A_5, A_{14} and A_{16} is independently.
20   selected from the group consisting of I, V, NLe, L and F;
        and/or wherein each of A_7 and A_{12} is independently
selected from the group consisting of I, V, L, W, Y and F;
        and/or wherein A_{10} is R, G or P;
        and/or wherein A_{11} is R or W.
25

6.   The compound of claim 1 which is selected from the
group consisting of
        PG-1:   RGGRLCYCRRRFCVCVGR
        PG-2:   RGGRLCYCRRRFCICV
30   PG-3:   RGGGLCYCRRRFCVCVGR
        PG-4:   RGGRLCYCRGWICFCVGR
        PG-5:   RGGRLCYCRPRFCVCVGR
        PC-39:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-R
        PC-41:   R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G
35   PC-100: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-Y

PC-101: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-T

PC-102: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-A

PC-103: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-L

PC-104: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-I

PC-105: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-F

PC-106: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W

PC-108: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R

R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-R

R-G-G-R-L-C-W-C-R-R-R-F-C-V-C-V-G-R

R-G-G-R-L-C-Y-C-R-R-R-W-C-V-C-V-G-R

R-G-G-R-L-C-Y-C-R-R-R-F-C-W-C-V-G-R

R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-W-G-R

IB-247: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH

IB-249: R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-OH

IB-223: R-G-G-G-L-C-Y-C-R-R-G-F-C-V-C-F-G-R

IB-224: R-G-G-G-L-C-Y-C-R-R-P-F-C-V-C-V-G-R

IB-324: R-G-G-G-L-C-Y-C-R-P-R-F-C-V-C-V-G-R-OH

IB-341: R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R-OH (X=NMeG)

IB-342: R-G-G-R-L-C-Y-C-R-X-R-F-C-V-C-V-G-R (X=NMeG)

IB-384: R-G-G-R-L-C-Y-C-X-G-R-F-C-V-C-V-G-R (X=Cit)

IB-398: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R

IB-399: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-G-R-OH

IB-218: R-G-G-G-L-C-Y-C-F-P-K-F-C-V-C-V-G-R

IB-349: R-G-G-R-L-C-Y-C-R-X-R-Cha-C-V-C-W-G-R (X=NMeG)

IB-350: R-G-G-R-W-C-V-C-R-X-R-Cha-C-Y-C-V-G-R (X=NMeG)

IB-394: R-G-G-R-W-C-V-C-R-G-R-Cha-C-Y-C-V-G-R

IB-416: R-G-G-R-L-C-Y-C-R-R-R-F-C-NMeV-C-V-G-R

IB-400: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V

IB-401: R-G-G-R-V-C-Y-C-R-G-R-F-C-V-C-V-OH

PC-49: R-G-G-R-L-C-W-A-R-R-R-F-A-V-C-V-G-R

PC-50: R-G-G-R-L-C-Y-A-R-R-R-W-A-V-C-V-G-R

PC-52: R-G-G-R-L-A-W-C-R-R-R-F-C-V-A-V-G-R

PC-53: R-G-G-R-L-A-Y-C-R-R-R-F-C-V-A-W-G-R

PC-55: R-G-G-R-L-A-W-A-R-R-R-F-A-V-A-V-G-R

PC-56: R-G-G-R-L-A-Y-A-R-R-R-W-A-V-A-V-G-R

PC-57: R-G-G-R-L-A-Y-A-R-R-R-F-A-V-A-W-G-R

IB-214: R-G-G-G-L-C-Y-A-R-G-W-I-A-F-C-V-G-R

IB-216: R-G-G-G-L-C-Y-A-R-G-F-I-A-V-C-F-G-R

```
IB-225:  R-G-G-G-L-C-Y-A-R-P-R-F-A-V-C-V-G-R
IB-226:  R-G-G-G-L-C-Y-T-R-P-R-F-T-V-C-V-G-R
IB-227:  R-G-G-G-L-C-Y-A-R-K-G-F-A-V-C-V-G-R
IB-288:  R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R-OH
IB-289:  R-G-G-R-L-C-Y-A-R-R-R-F-A-V-C-V-G-R
```

and the amidated forms thereof either in linear or cystine-bridged form.

7.   The compound of any of claims 1-6 wherein all amino acids are in the D-configuration.

8.   A recombinant expression system for production of an antimicrobial peptide having the amino acid sequence of the compound of any of claims 1-6 which expression system comprises a nucleotide sequence encoding said peptide operably linked to control sequences for effecting expression.

9.   A recombinant host cell modified to contain the expression system of claim 8.

10.   A method to produce an antimicrobial or antiviral peptide or intermediate peptide therefor which method comprises culturing the modified host cells of claim 9 under conditions wherein said peptide is produced; and recovering the peptide from the culture.

11.   The method of claim 10 which further comprises effecting cystine linkages of said peptide and/or modifying the N-terminus and/or C-terminus of said peptide.

12.   A pharmaceutical composition for antimicrobial or antiviral use which comprises the compound of any of claims 1-7 in admixture with at least one pharmaceutically acceptable excipient.

13. A composition for application to plants or plant environments for conferring resistance to microbial or viral infection in plants which comprises the compound of any of
5   claims 1-7 in admixture with at least one environmentally acceptable diluent.

14. A method to prevent the growth of a virus or microbe which method comprises contacting a composition
10 which supports the growth of said virus or microbe with an amount of the compound of any of claim 1-7 effective to prevent said growth.

15. A method to inactivate the endotoxin of gram-
15 negative bacteria, which method comprises contacting said endotoxin with an amount of the compound of any of claims 1-7 effective to inactivate said endotoxin.

16. Antibodies specifically reactive with the compound
20 of any of claims 1-7.

FIG. 1

o E. COLI

L. MONOCYTOGENES

△ C. ALBICANS

FIG. 2

FIG. 3

E. COLI ML—35p

▼ NP—1
□ HNP1
○ PG—1
● PG—2
▽ PG—3

FIG. 4a

LISTERIA MONOCYTOGENES EGD

▼ NP—1
□ HNP1
○ PG—1
● PG—2
▽ PG—3

FIG. 4b

SUBSTITUTE SHEET (RULE 26)
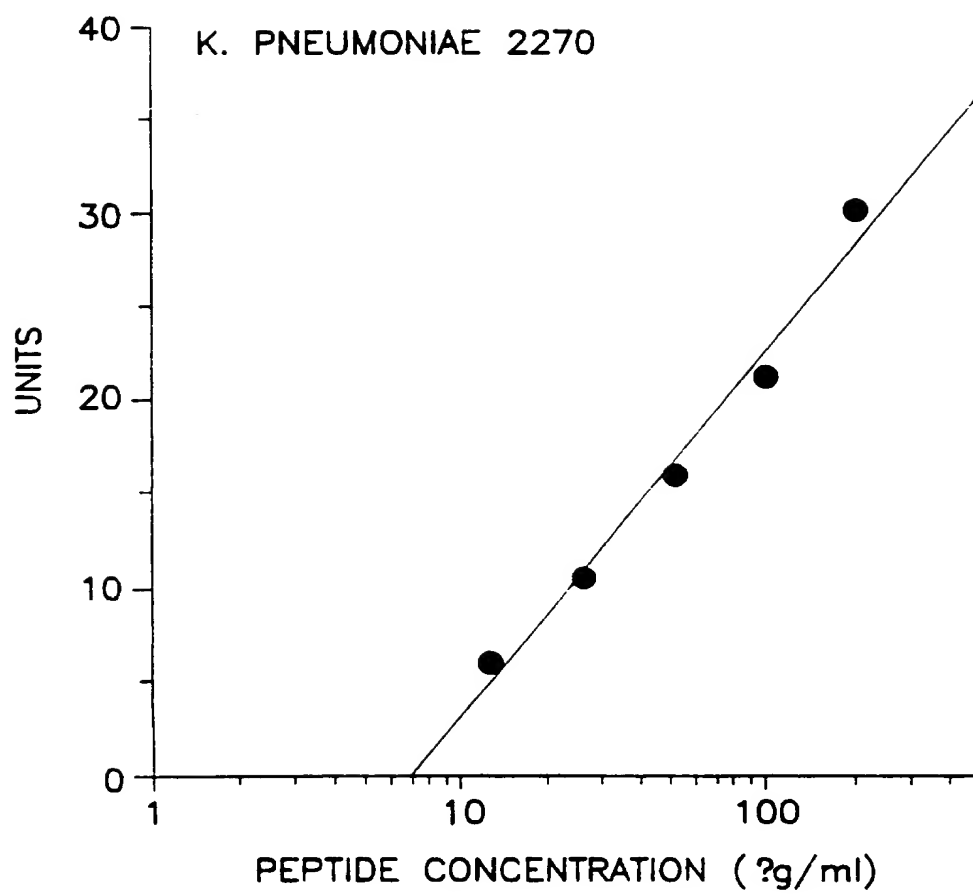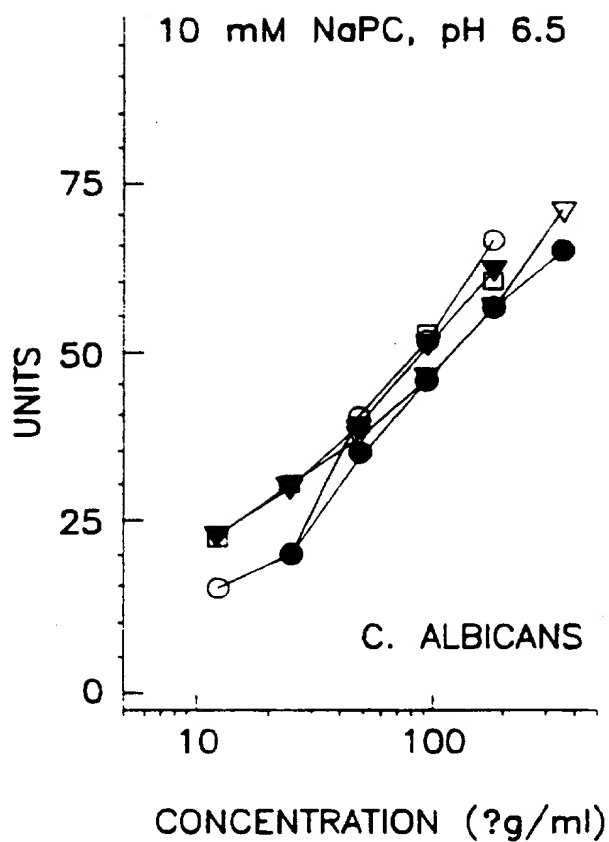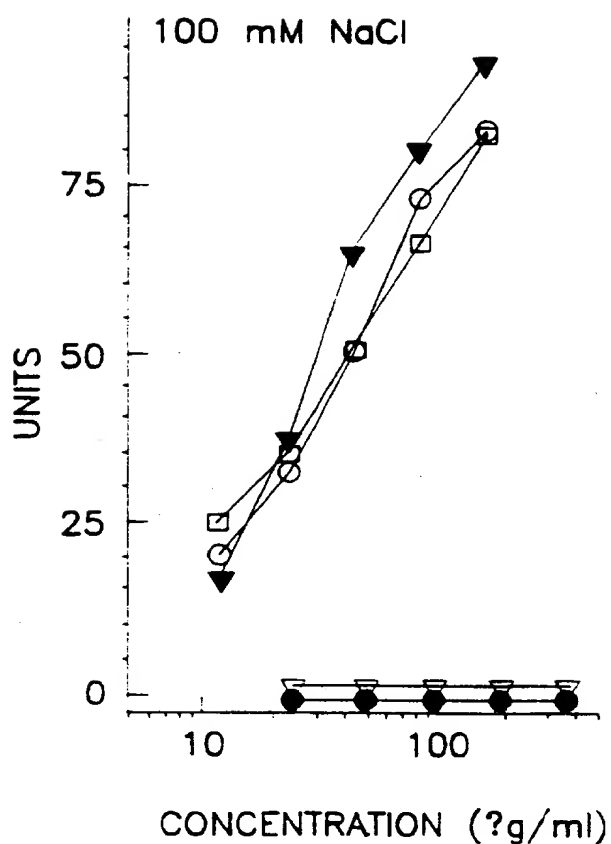
CANDIDA ALBICANS

▼ NP−1
□ HNP1
○ PG−1
   PG−2
▽ PG−3

FIG. 4c

STAPH AUREUS 930918−3

FIG. 4d

FIG. 4e

FIG. 5a-I

FIG. 5a-2

10 mM NaPC, pH 6.5

+ 100 mM NaCl

FIG. 5b-I

FIG. 5b-2

FIG. 5c-1

FIG. 5c-2

FIG. 6a

FIG. 6b

L. MONOCYTOGENES

CONCENTRATION (?g/ml)
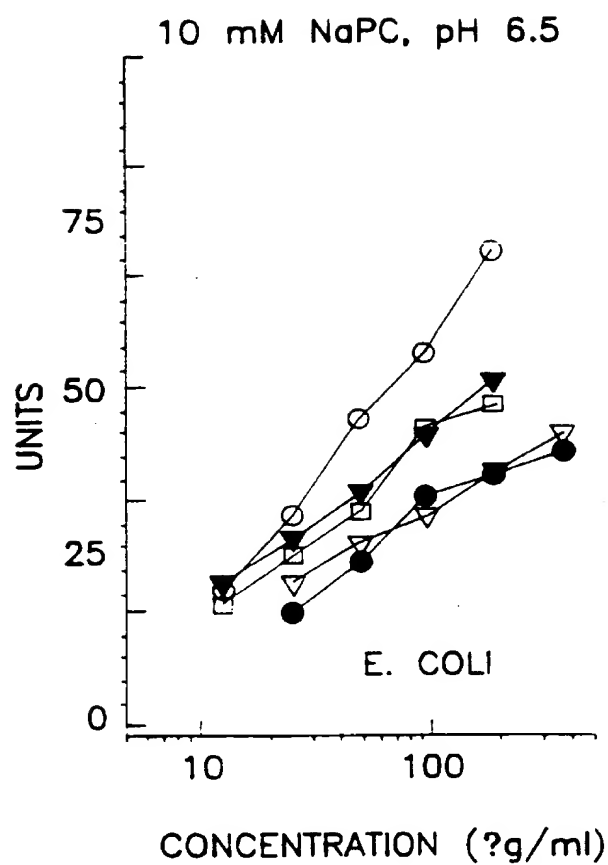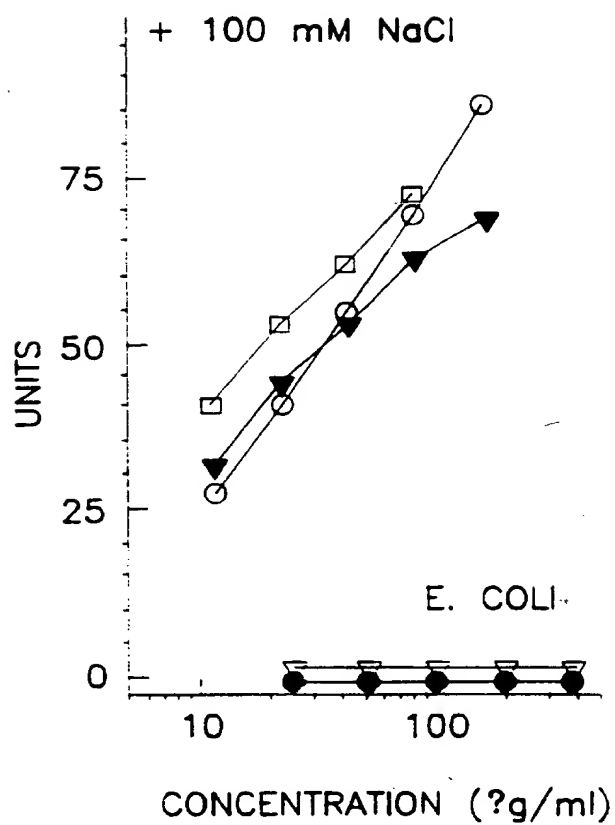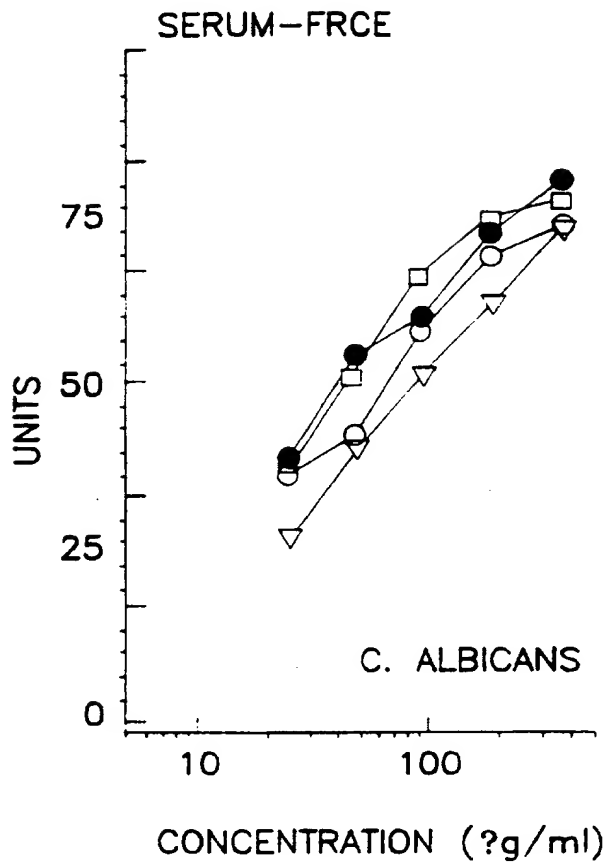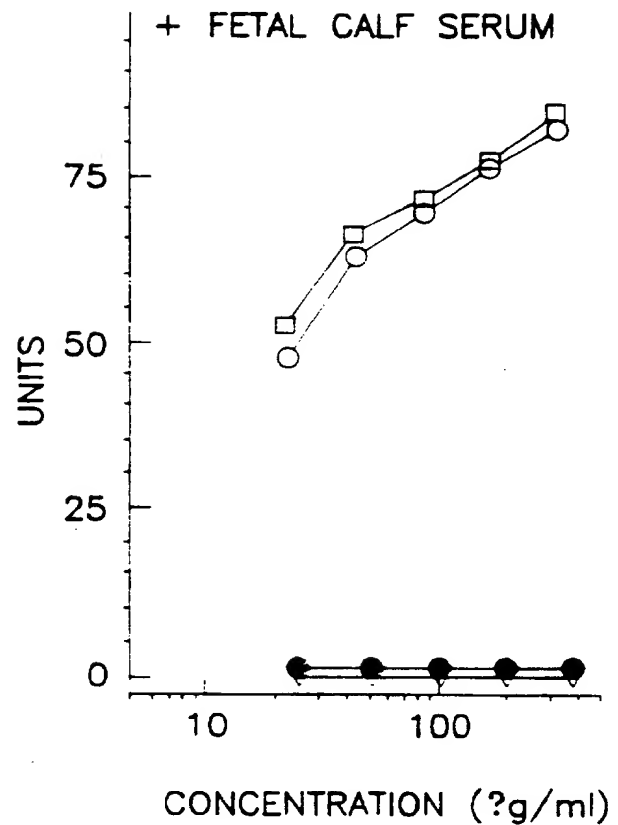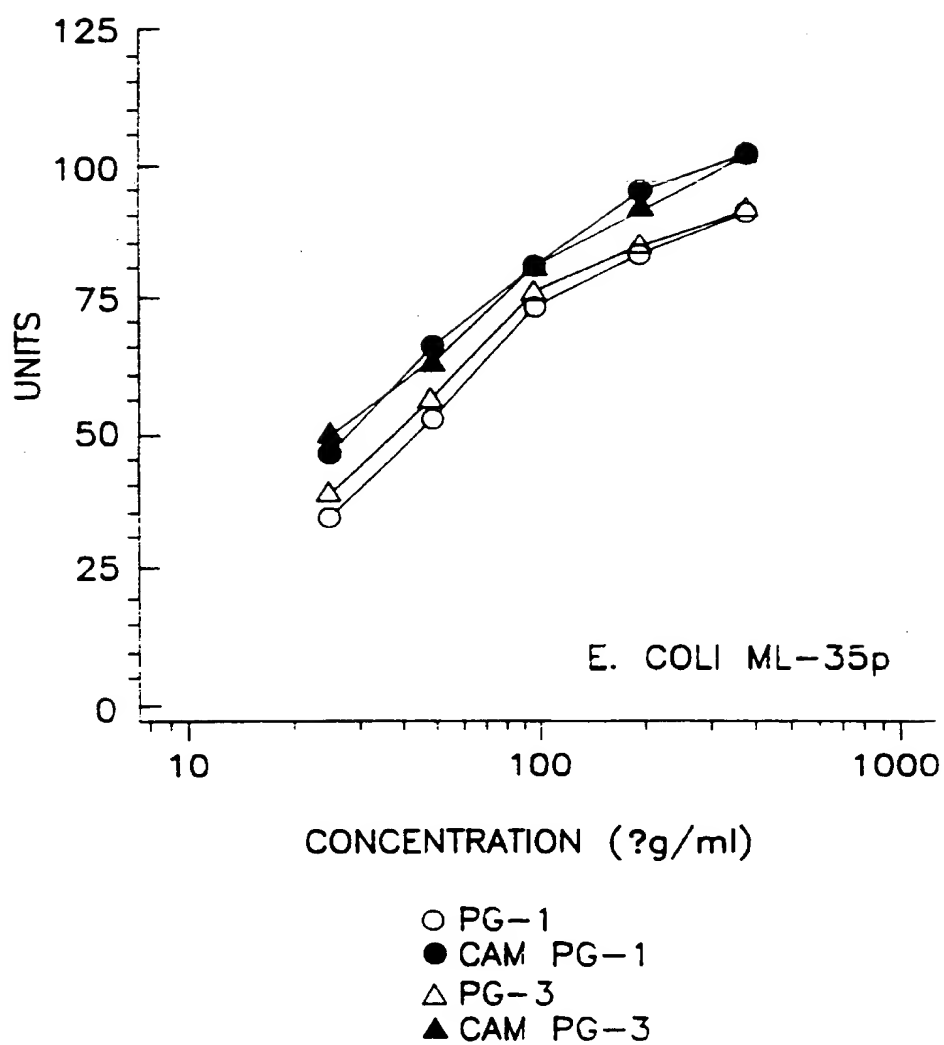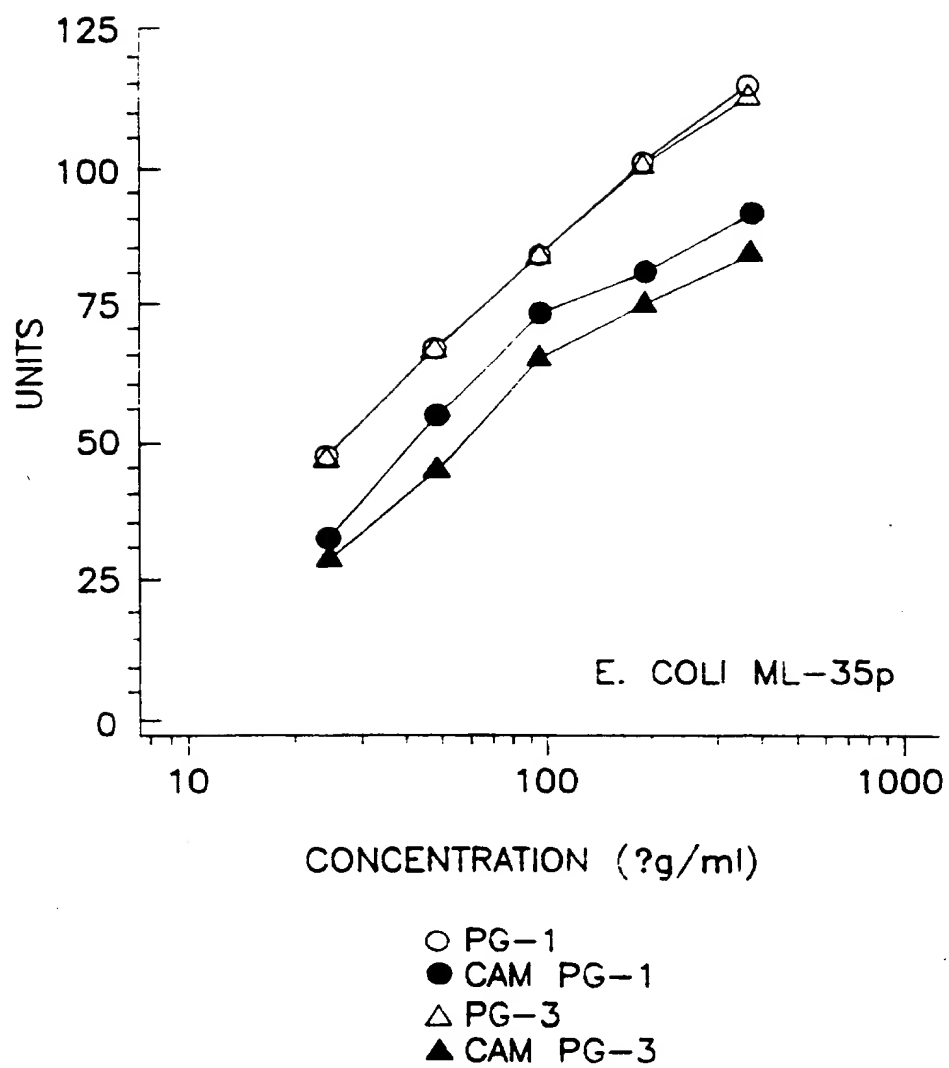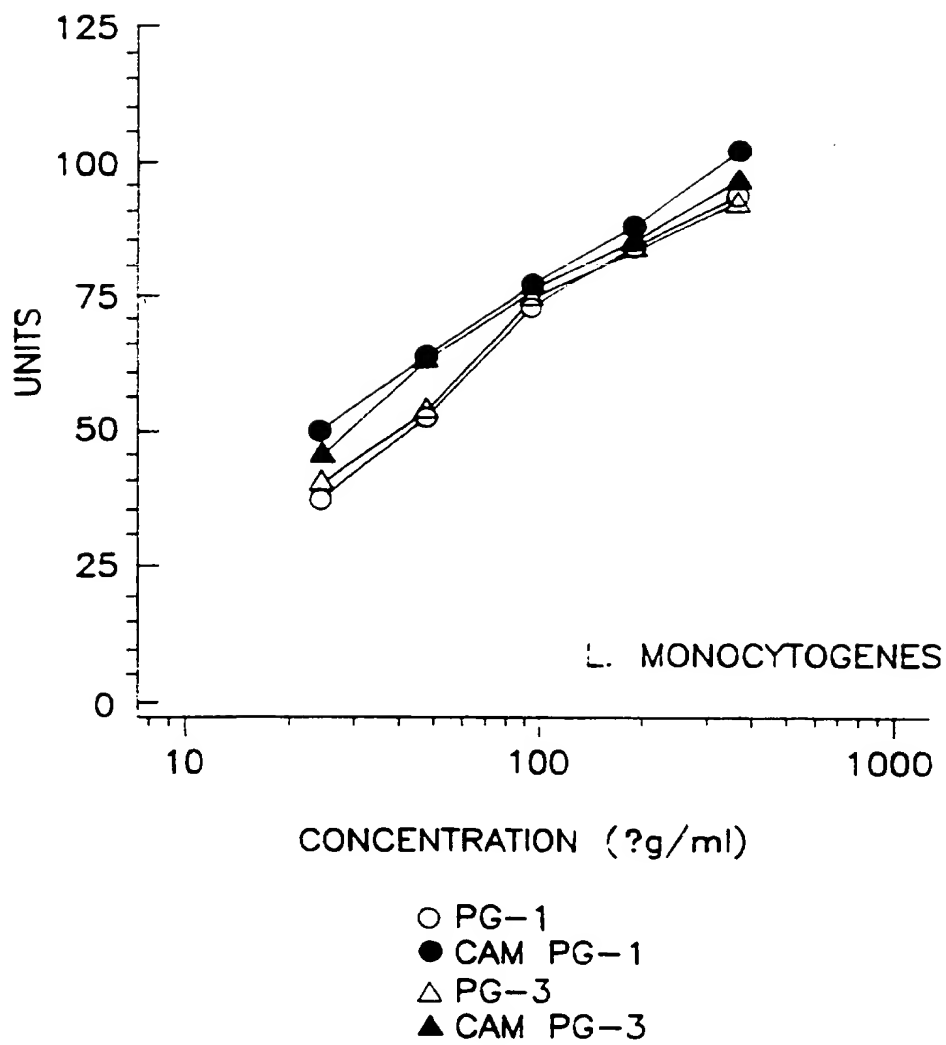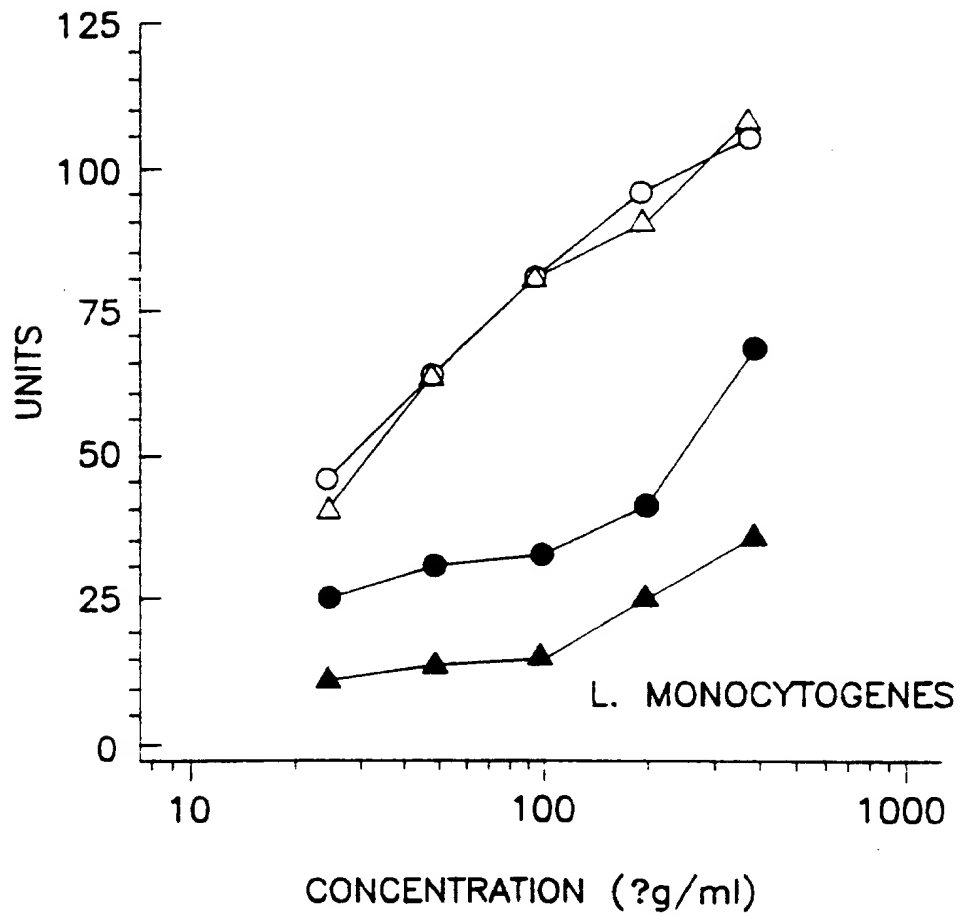
○ PG—1
● CAM PG—1
△ PG—3
▲ CAM PG—3

FIG. 6c

FIG. 6d

PEPTIDE CONCENTRATION ADDED TO WELL (?g/ml)

○ NATIVE PG—1
● CAM PG—1
□ NATIVE PG—3
■ CAM PG—3

FIG. 6e

PEPTIDE CONCENTRATION ADDED TO WELL (?g/ml)
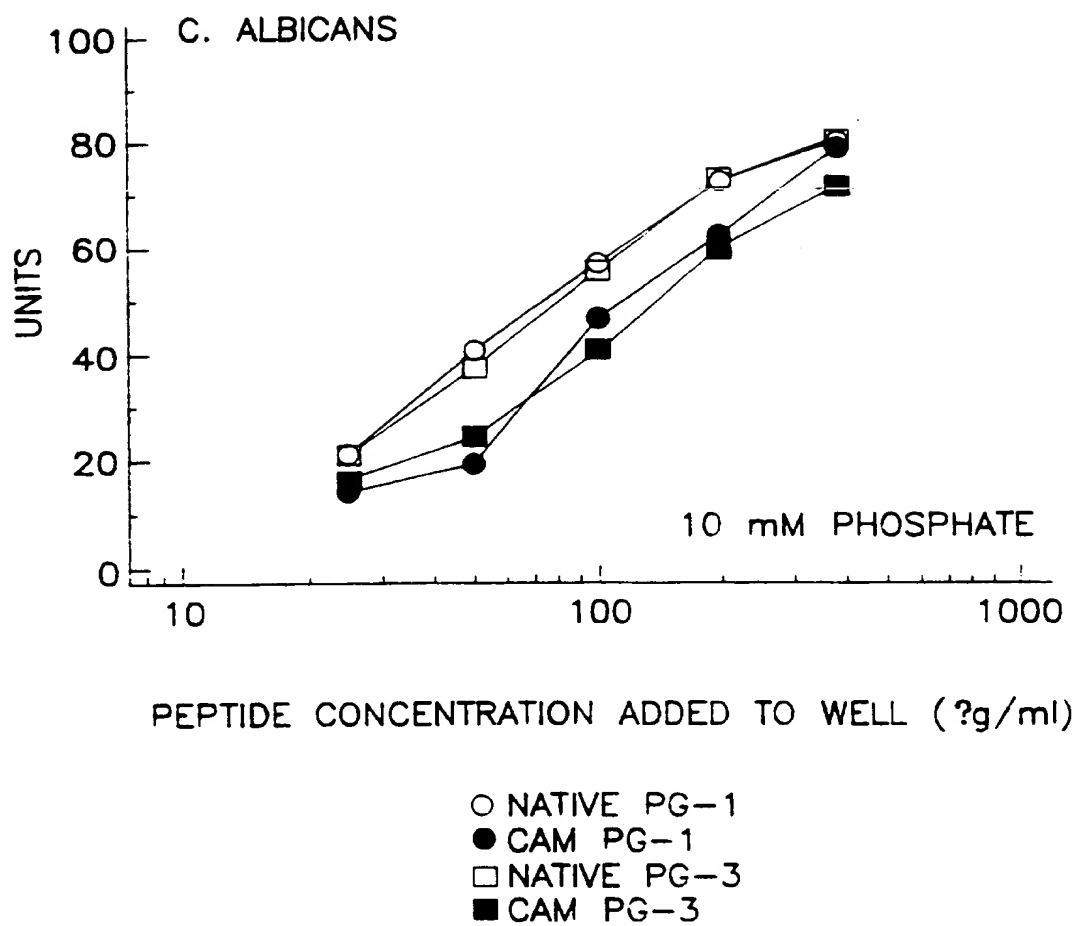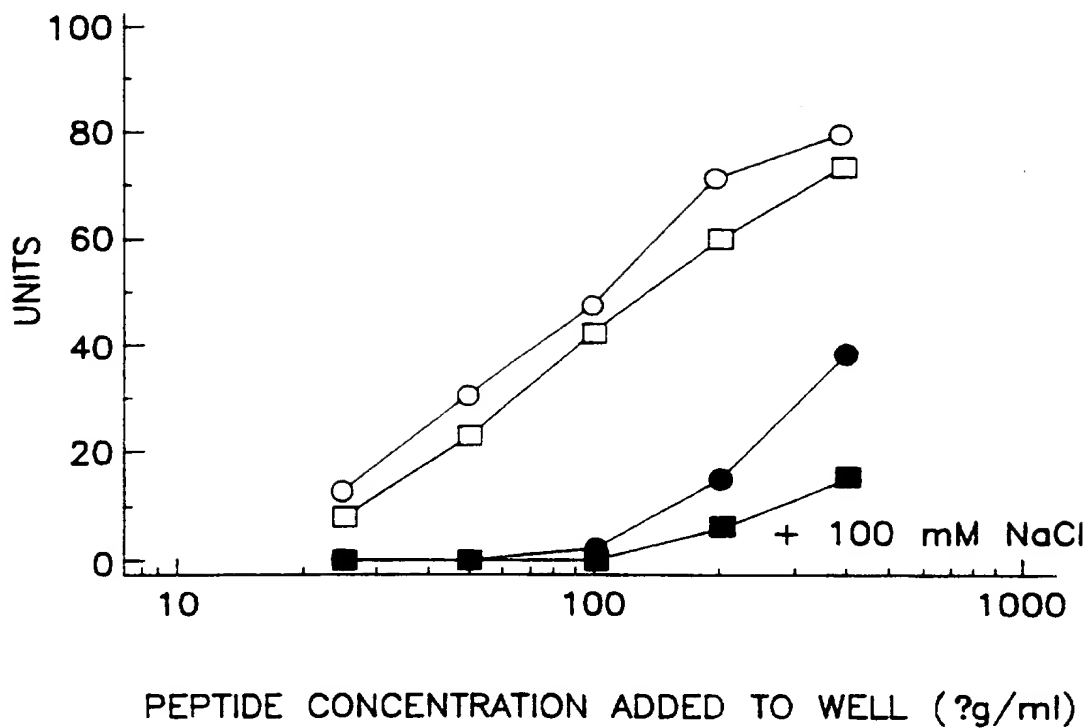
O NATIVE PG−1
● CAM PG−1
□ NATIVE PG−3
■ CAM PG−3

FIG. 6f

FIG. 9

```
               10        20        30        40        50
ATGGAGACCGAGAGAGCCAGCCTGTGCCTGGGGCGCTGGTCACTGTGGCTTCTGCTGCTG    60
MetGluThrGlnArgAlaSerLeuCysLeuGlyArgTrpSerLeuTrpLeuLeuLeuLeu    20

GCACTCGTGGTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTACAGGGAGGCCGTGCTTCGT   120
AlaLeuValValProSerAlaSerAlaGlnAlaLeuSerTyrArgGluAlaValLeuArg    40

GCTGTGGATCGCCTCAACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCTCCTGGAGCTG   180
AlaValAspArgLeuAsnGluGlnSerSerGluAlaAsnLeuTyrArgLeuLeuGluLeu    60

GACCAGCCGCCCAAGGCCGACGAGGACCCGGGCACCCCGAAACCTGTGAGCTTCACGGTG   240
AspGlnProProLysAlaAspGluAspProGlyThrProLysProValSerPheThrVal    80

AAGGAGACTGTGTGTCCCAGGCCGACCCGGCAGCCCCCGGAGCTGTGTGACTTCAAGGAG   300
LysGluThrValCysProArgProThrArgGlnProProGluLeuCysAspPheLysGlu   100

AACGGGCGGGTGAAACAGTGTGTGGGGACAGTCACCCTGGATCAGATCAAGGACCCGCTC   360
AsnGlyArgValLysGlnCysValGlyThrValThrLeuAspGlnIleLysAspProLeu   120

                                          G³                G⁴          --
GACATCACCTGCAATGAGGTTCAAGGTGTCAGGGGAGGTCGCCTGTGCTATTGTAGGCGT   420
AspIleThrCysAsnGluValGlnGlyValArgGlyGlyArgLeuCysTyrCysArgArg   140
                                      Gly³              Gly⁴

           A²
T⁴ A⁴      T⁴              T²
AGGTTCTGCGTCTGTGTCGGACGAGGATGACGGTTGCGACGGCAGGCTTTCCCTCCCCCA   480
ArgPheCysValCysValGlyArgGly---                               149

Trp⁴Ile⁴ Phe⁴              ---²
         Ile²

ATTTTCCCGGGGGCCAGGTTTCCGTCCCCCAATTTTTCCGCCTCCACCTTTCCGGCCCGCA   540

                                  A²  G²
CCATTCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAGGATTTGCAGGCAACTCACCCAG   600

        C⁴
AAGGCCTTTCGGTACATTAAAATCCCAGCAAGGAGACCTAAGCATCTGCTTTGCCCAGGC   660

CCGCATCTGTCAAATAAATTCTTGTGAAACC                              691
```

FIG. 7

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```
ATGGAGACCCAGAGAGCCAGCCTGTGCCTGGGGCGCTGGTCACTGTGGCTTCTGCTGCTG    60
 M  E  T  Q  R  A  S  L  C  L  G  R  W  S  L  W  L  L  L  L
G5
GCACTCGTGGTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTACAGGGAGGCCGTGCTTCGT   120
 A  L  V  V  P  S  A  S  A  Q  A  L  S  Y  R  E  A  V  L  R
G5
GCTGTGGATCGCCTCAACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCTCCTGGAGCTG   180
 A  V  D  R  L  N  E  Q  S  S  E  A  N  L  Y  R  L  L  E  L
GACCAGCCGCCCAAGGCCgtgagtcgggcagggggctcaggagggggctgggggcgggggc   240
 D  Q  P  P  K  A
tgtcccccacccgccccggggctccctgtccctccccctgctcaggctgtccctcctgcc   300
aggaaggcacttgtccctctaaggggggacccctctgccaggaaaccttcccagagctgg   360
gtgccctgcccgcgtgagagcttcccgccttagcctctgggctgtgggctcagggccctg   420
cacagcctgtgaggcaggagcgggctctgtcccctccctgtgcacccagcaccaagccc...480
agggccaggctcccagcagggggctgcagaggctgctgtctaggtgggggcggggagggg   540
tgacagatccgaggggggaagcctgagcccgagtcccatctccccactttgatccttgacc   600
                               A5
agGACGAGGACCCGGGCACCCCGAAACCTGTGAGCTTCACGGTGAAGGAGACTGTGTGTC   660
  D  E  D  P  G  T  P  K  P  V  S  F  T  V  K  E  T  V  C
CCAGGCCGACCCGGCAGCCCCCGGAGCTGTGTGACTTCAAGGAGAACGGGgtgaggctgg   720
 P  R  P  T  R  Q  P  P  E  L  C  D  F  K  E  N  G
gggctgggggcgctggcggatgcttcccaaggagctgaacaggagagcctgctggggaag   780
atgtccaggccctggggtgaggctgggagctcatggatggaggaggggggtcccagttt   840
                 t3
gaccttgagtctcccccttccagCGGGTGAAACAGTGTGTGGGGACAGTCACCCTGGATCA   900
                       R  V  K  Q  C  V  G  T  V  T  L  D  Q
GATCAAGGACCCGCTCGACATCACCTGCAATGAGgtgagtggcccccttattggtgtcaag   960
 I  K  D  P  L  D  I  T  C  N  E
ttgctaatgggttggtgtggggaactccttgggagtgttacccgctgccccatccaggc   1020
gtggaaaggcccctctaccccggcccttccctcacctcggccccagggctccaggtctgg   1080
ctctgtcatccttagggccgcggttccctcaatggggtcccccctcgtatttgtcagaa   1140
                                          g3,5
aggcacatttcaggcccccaccccgaccctctgaatcacactcttgggtggagcccagcct   1200
tgtctcttctcccaagatcccagcgggttcttcctgtgctgtcggctgagaggcagtgac   1260
cggactaatggacttgcaggccctgctcctggccagctttgcggggctgggtttgggacc   1320
ctggcaaggccccagccatctctgggcctgagtccacttatgtgtctgtggggattcaa   1381
                              g3,5
                              t5
ccacgtgctccaaaggtcacagccagaggtggaccagggccccaagcctcttactgtttc   1440
```

FIG. 8a

```
cccattcagggattttttctagtctggagggagggttcttgtcttgacccttggccagacc          1500
                                                       G3
ccacccgaaacctgtttctcttggtcacagGTTCAAGGTGTCAGGGGAGGTCGCCTGTGC          1560
                               F  Q  G  V  R  G  G  G  R  L  C
                                                      G3
                 C5                          T5
TATTGTAGGCGTAGGTTCTGCGTCTGTGTCGGACGAGGATGACGGTTGCGACGGCAGGCT          1620
 Y  C  R  R  R  F  C  V  C  V  G  R  G  ***
                    P5
TTCCCTCCCCCAATTTTCCCGGGGCCAGGTTTCCGTCCCCCAATTTTTCCGCCTCCACCT          1680

TTCCGGCCCGCACCATTCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAGGATTTGCAGG          1740
                        C3,5
CAACTCACCCAGAAGGCCTTTCGGTACATTAAAATCCCAGCAAGGAGACCTAAGCATCTG          1800

CTTTGCCCAGGCCCGCATCTGTCAAATAAATTCTTGTGAAACC                          1843
```

FIG. 8b

FIG. 10

FIG. IIa-2

FIG. IIa-I

FIG. IIb-2

FIG. IIb-I

FIG. IIc-2

FIG. IIc-I

FIG. 12b

FIG. 12a

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FIG. 12d

○ PG—1
● ENANTIO PG—1

FIG. 12c

○ NATIVE PG—1
● + TRYPSIN
△ + CHYMOTRP.

OPEN SYMBOLS = KITE, CLOSED SYMBOLS = BULLET

FIG. 13a

FIG. 13b
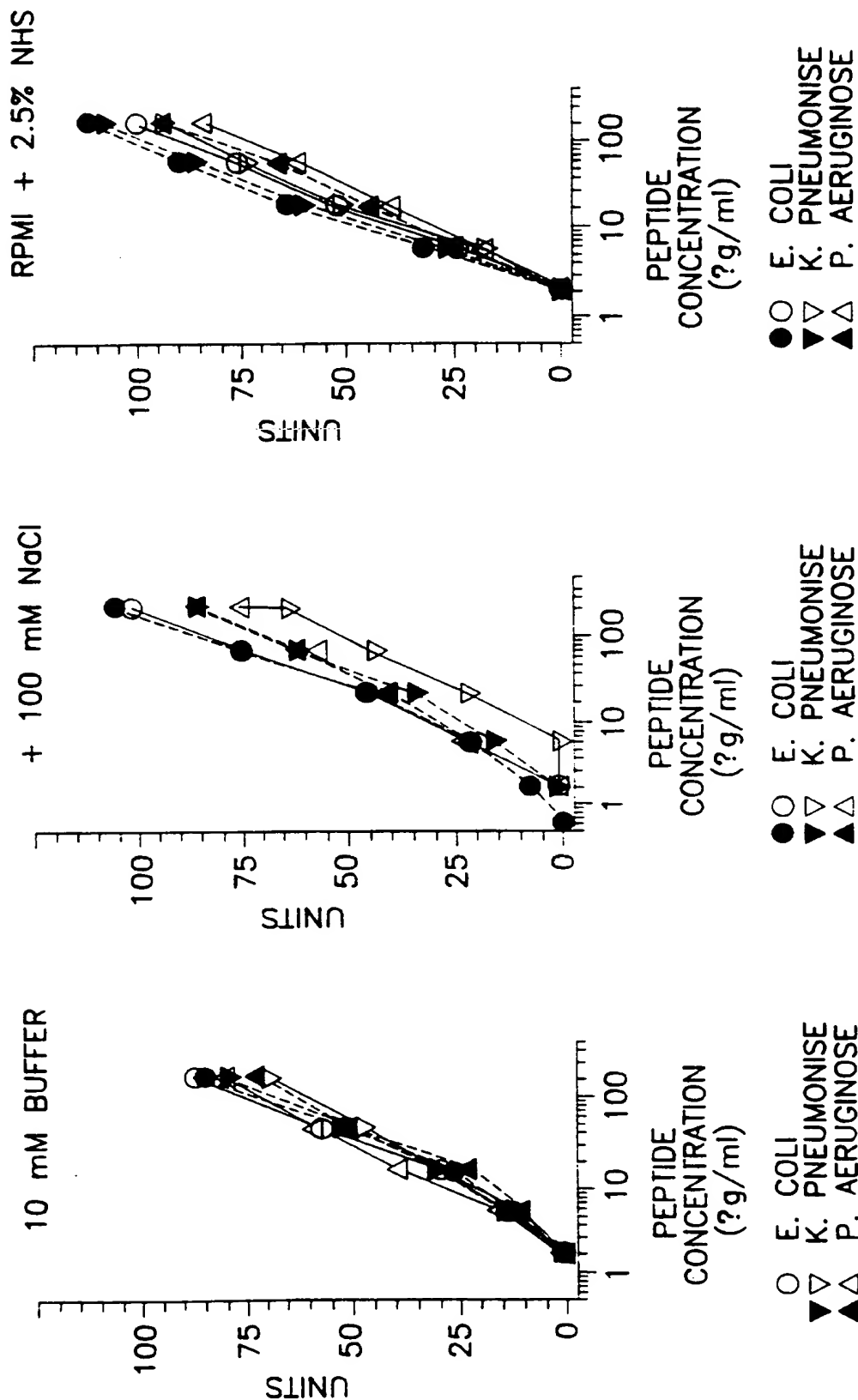
FIG. 13c

OPEN SYMBOLS = KITE, CLOSED SYMBOLS = BULLET

10 mM BUFFER

PEPTIDE CONCENTRATION (?g/ml)

UNITS

○ E. COLI
▽ K. PNEUMONISE
△ P. AERUGINOSE
▼▲ (closed)

FIG. 14a

+ 100 mM NaCl

PEPTIDE CONCENTRATION (?g/ml)

UNITS

●○ E. COLI
▼▽ K. PNEUMONISE
▲△ P. AERUGINOSE

FIG. 14b

RPMI + 2.5% NHS

PEPTIDE CONCENTRATION (?g/ml)

UNITS

○ E. COLI
▽ K. PNEUMONISE
△ P. AERUGINOSE
● ▼ ▲ (closed)
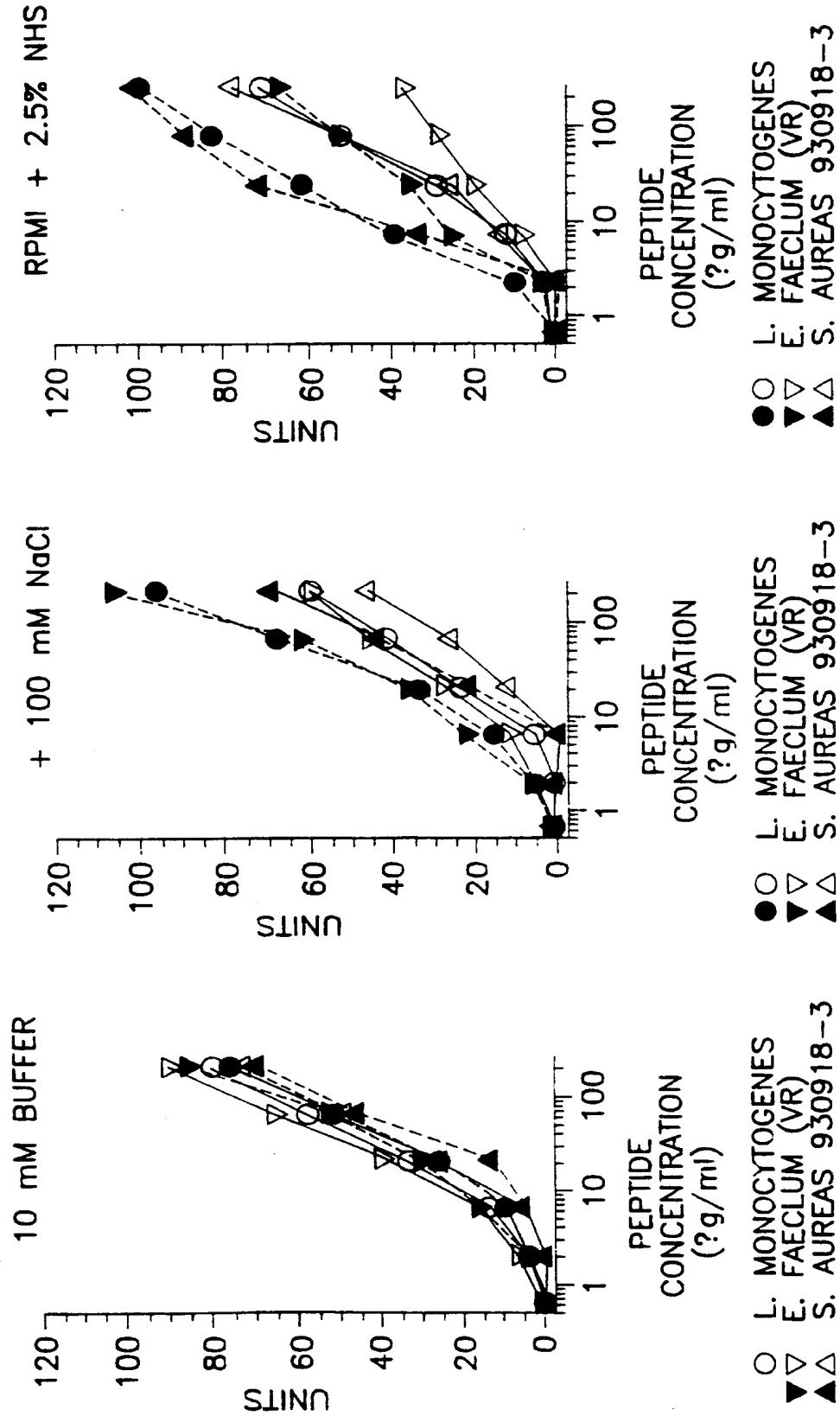
FIG. 14c
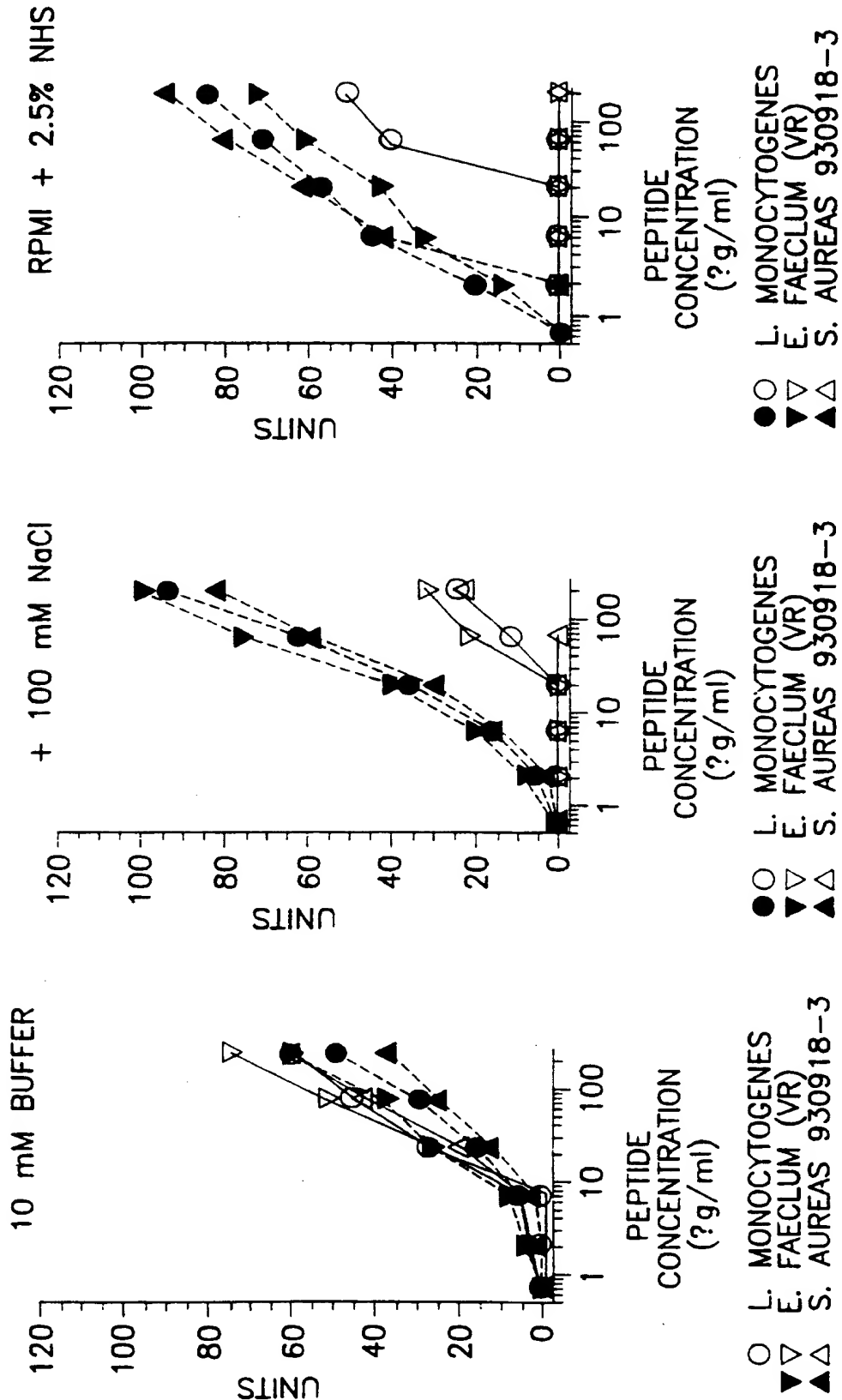
FIG. 15a

FIG. 15b

FIG. 15c

OPEN SYMBOLS = LINEARIZED, CLOSED SYMBOLS = sPG—1

FIG. 16a

FIG. 16b

FIG. 16c

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/00, 7/08; A61K 38/10; C12N 15/12, 15/74
US CL :514/12, 13; 435/69.1, 320.1; 530/324, 325, 326; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 13; 435/69.1, 320.1; 530/324, 325, 326; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOKRYAKOV et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Letters. July 1993, Volume 327, Number 2, pages 231-236, see entire document.	1, 2, 5, 6
A	WO 89/11291 A1 (INVITRON CORPORATION) 30 November 1989, pages 7-12, 14 and 15.	1, 2, 5, 6
A	EP 0 545 730 A1 (PIONEER HI-BRED INTERNATIONAL, INC.) 09 June 1993, pages 5-8.	1
Y	WO 93/24139 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 09 December 1993, see pages 17-22.	1 and 2

[X] Further documents are listed in the continuation of Box C.	[ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 SEPTEMBER 1996	1 1 OCT 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	*William W. Moore*
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94/21672 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 29 September 1994, see pages 6-20, 28, 29 and 34-36.	1, 2, 5, 6
Y	WO 95/10534 A1 (SEIKAGAKU CORPORATION) 20 April 1995, see pages 4-30.	1-3, 5, 6
X	MIRGORODSKAYA et al. Primary structure of three cationic peptides from porcine neutrophils. FEBS Letters, September 1993, Volume 330, Number 3, pages 339-342, see page 341.	1, 2, 5, 6
X	STORICI et al. A novel cDNA sequence encoding a pig leukocyte antimicrobial peptide with a cathelin-like pro-sequence. Biochemical and Biophysical Research Communications. 15 November 1993, Volume 196, No. 3, pages 1363-1368, see pages 1364-1364 and Figures 1 and 2.	1, 2, 5, 6
X -- Y	ZHAO et al. Identification of a new member of the protegrin family by cDNA cloning. FEBS Letters. June 1994, Volume 346, pages 285-288, see pages 286-288 and Figures 1-4.	1, 2, 6 --------- 5
X	WO 95/03325 A1 (UNIVERSITY OF CALIFORNIA, LOS ANGELES) 02 February 1995, see entire document.	1-6
A	MATSUZAKI et al. Role of Disulfide Linkages in Tachyplesin-Lipid Interactions. Biochemistry. November 1993, Volume 32, pages 11704-11710.	1-5
A	TAMAMURA et al. A comparative study of the solution structures of tachyplesin I and a novel anti-HIV synthetic peptide, T22 ([Tyr5,12, Lys7]-polyphemusin II), determined by nuclear magnetic resonance. Biochimica et Biophysica Acta. May 1993, Volume 1163, pages 209-216.	1, 2 and 5
A	TAMAMURA et al. Antimicrobial Activity and Conformation of Tachyplesin I and Its Analogs. Chemical and Pharmaceutical Bulletin. May 1993, Volume 41, No. 5, pages 978-980.	1, 2, 4, 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/07594

Box I Observati ns where certain claims were found unsearchable (C ntinuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-16
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Pr test ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)✶

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

A-GeneSeq23, PIR47, Swiss-Prot32 & issued U.S. Patent protein databases were searched with SEQ IDs NOs:2, 4, 6, 8, and 10-67 of the latter priority document; DIALOG databases Medline, CA Seach, Agricola, Biosis Previews, Derwent Biotech Abs., Current Biotech Abs. and Derwent World Patent Index were searched with terms defensin, corticostatin, protegrin, tachyplesin, cryptdin, cecropin, magainin and antimicrobial peptide